

THE FUNCTION OF THE INSECT OCELLUS

By D. A. PARRY, *From the Department of Zoology, University of Cambridge*

(Received 1 February 1947)

(With Three Text-figures)

INTRODUCTION

Many insects possess two types of eye: a compound eye and a simple eye or dorsal ocellus. Much work has been done on the powers of vision in insects, and it is known that these are almost entirely due to the presence and form of the compound eye. The perception of form and colour, and the appreciation of movement in the visual field, are its recognized functions.

No such clearly defined functions have yet been assigned to the dorsal ocellus. The optical system of this organ is such that no image can be formed on the retina. Its occurrence within the class is extremely erratic and bears no apparent relation to the mode of life or the systematic position of those insects in which it occurs. On the other hand, insects deprived of their ocelli show certain peculiarities of behaviour, which suggest that the ocellus *is* in some way concerned in the response of the animal to light.

The object of the present research was to obtain more knowledge of the function of the ocellus, primarily by recording the nervous response to ocellar stimulation. The migratory locust (*Locusta migratoria migratoroides*) provides a preparation just large enough for electrodes to be placed on the ocellar nerve and electrical changes occurring there to be amplified and recorded. It has also proved possible to record from the circum-oesophageal commissure and thus to investigate, at a point nearer the motor system, the effects of stimulating the ocellus and cutting the ocellar nerves.

Information has also been obtained on the distribution of the ocellus among insects, the optical system of the ocellus, and the histology of the ocellar nerve.

THE OCCURRENCE OF THE OCELLUS

An examination of the literature and of museum collections was made to determine the occurrence of the ocellus within the class of insects, as it was hoped that this would throw some light on the function of the organ. In fact its distribution seemed quite anomalous, and emphasized, rather than helped to solve, the problem. The following list summarizes the facts.

ORTHOPTERA: always present in Acrididae and Gryllidae; sometimes present in Blattidae, Mantidae, Tettigoniidae; not present in Grylloblattidae. DERMAPTERA: absent. PLECOPTERA: two or three present. ISOPTERA: present. EMBIOPTERA: absent. PSOCOPTERA: sometimes present. ANOPLURA: absent. EPHEMEROPTERA: present. ODONATA: usually present. THYSANOPTERA: present. HEMIPTERA: great variation. Some families separated on the presence or absence of ocelli. Several families in which some genera possess ocelli and some do not. NEUROPTERA: conspicuous in some families, absent in others. MECOPTERA: some genera with ocelli, others without. TRICHOPTERA: some families with ocelli, others

without. One family including six genera with ocelli and two without. LEPIDOPTERA: sometimes present. COLEOPTERA: absent except in a few species not all in the same family. STREPSIPTERA: absent. HYMENOPTERA: usually present, but sometimes absent in the Vespoidea. DIPTERA: sometimes present. APHANIPTERA: uncertain.

This list shows the general irregularity of the distribution. From a more detailed analysis of the orders (especially Orthoptera and Hemiptera) it frequently appeared that of two insects, very closely related systematically and with apparently exactly the same habits, one may possess well-developed ocelli and the other have lost them altogether.

THE STRUCTURE AND OPTICAL SYSTEM OF THE OCELLUS

There is little variation in the structure of the ocellus throughout the class. The lens is part of the exoskeleton. The corneagen layer is a transparent area of the hypodermis underlying, and secreting, the lens. The retina consists of pigment cells and sense cells, the latter grouped together to form rhabdomes. In some ocelli the retina is a regular columnar epithelium, while in others, as in the typical orthopteran ocellus, its constituent cells are more irregularly arranged. The sense cells bear only short processes extending to the synaptic region immediately behind the retina.

The close proximity of lens and retina precludes the possibility of image perception. Homann (1924) investigated living and prepared ocelli of many insects and showed that although the lens was sufficiently well formed to produce an image, this would fall a considerable distance behind the retina. Homann did not study *L. migratoria*, but his general conclusion was confirmed by a determination of the distance of the principal focus and the retina from the front surface of the lens, which was made during the present work.

Following Homann, the principal focus was found by examining the ocellus through a microscope to the tube of which was secured a horizontal cross-piece bearing a point source of light at either end. The images of these lights were reflected in the front surface of the lens, and their distance apart could be measured by a micrometer. Knowing the actual distance between the lights, and the vertical distance between them and the ocellar lens, the radius of curvature of the latter was calculated. The position of the principal focus was then worked out, knowing the refractive index of the lens. The value thus obtained took no account of refraction at the inner surface of the lens, but as the refractive index of lens and corneagen layer cannot be very different, this simplification will not introduce serious error. The radius of curvature of the front surface of the lens was 0.23 mm., and the principal focus therefore at a point 0.44 mm. behind this surface (see Fig. 1).

The position of the retina was found directly. The animal was mounted below a microscope and a bright light thrown into the ocellus by a vertical illuminator. The lens and retina were brought into focus in turn and the distance between them measured by a micrometer. As the retina has a highly diffusing surface it was not very easy to focus, but among a large number of determinations the distance between the front of the lens and the retina was never found to reach the value of 0.09 mm. The principal focus was thus five times as far behind the lens as the retina, and so the possibility of image perception by the ocellus of *Locusta* may be excluded.

EXPERIMENTAL WORK

Previous work on the ocellus

The methods previously used to determine the function of the ocellus have all involved observing the effect of blinding upon the behaviour of the animal. Early investigators (Plateau, 1887-8; Homann, 1924; etc.) found that insects with their compound eyes painted over behaved as though blind whether they possessed ocelli or not. More recently, however, it has been shown that certain differences can be produced in the reactions of an insect towards light by painting over the ocelli, the compound eyes being left free. Thus *Drosophila* reacts more rapidly to a sudden exposure to light when the ocelli are free than when they are painted over (Bozler,

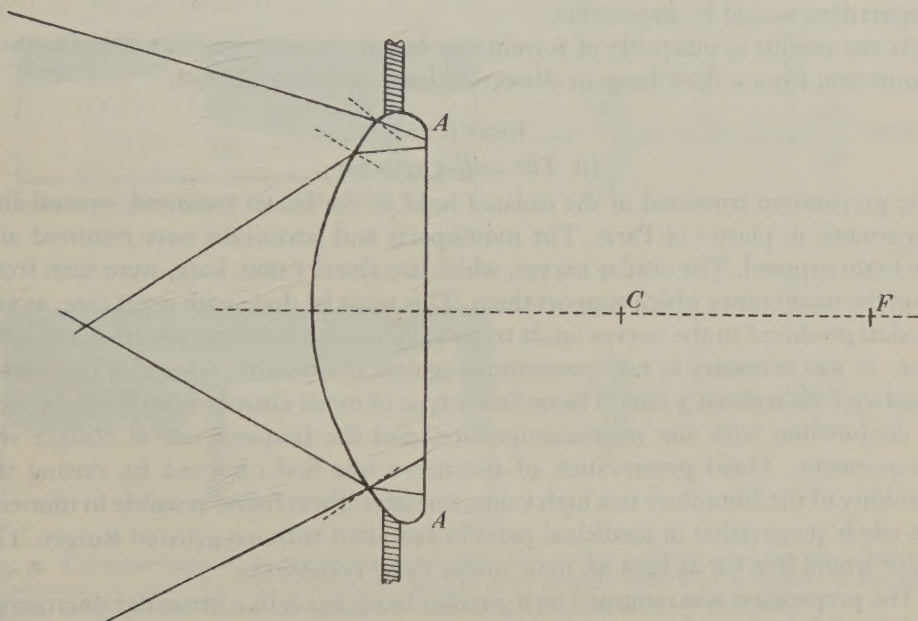


Fig. 1. Scale diagram of the ocellus. Light is shown incident on the lens from two distant point sources, and it is seen that the retina is uniformly illuminated by each except at its extreme edge. *AA*, outer face of retina; *C*, centre of curvature of outer face of lens; *F*, principal focus.

1925). Bees, walking along a path between two lights of equal intensity, may be caused to deviate towards one or other of the lights by painting the median and one lateral ocellus. The deviation occurs towards the side of the blinded ocellus (Müller, 1931). Bees which are normally negatively phototactic, at a temperature below 14° C., become positively so when the ocelli are painted over (Gotze, 1927; Müller, 1931).

In general it appears that ocelli are not capable of evoking a motor response by themselves, but only do so when working in co-operation with the compound eyes. They seem to 'prepare' the nervous system for the reception of stimuli from the more specific sense organs. This view has been elaborated by Wolsky (1930, 1931,

1933). He considers that whereas most sense organs possess a specific function in evoking certain motor responses and also have a general stimulating effect on the nervous system, the ocelli possess only the latter property.

METHODS

Almost all the present work was done on *Locusta migratoria*; the preparations used will be described in the appropriate sections. The amplifier was a three-stage directly coupled instrument with a balanced input and separate power stage driving a Matthews oscillograph. A fourth, condenser-coupled stage, could be switched into the circuit at will. During all this work a loud-speaker was continuously in use.

The fine platinum electrodes were moved into position by means of a Zeiss micromanipulator, without the use of which the investigation of these small preparations would be impossible.

As the ocellus is incapable of forming an image, its response to a simple on-off stimulation from a flash-lamp or dissection lamp was investigated.

RESULTS

(i) *The ocellar response*

The preparation consisted of the isolated head of the locust mounted, ventral side uppermost, in plaster of Paris. The mouthparts and tentorium were removed and the brain exposed. The ocellar nerves, which are about 1 mm. long, were then freed from the membranes which support them. This must be done with great care, as any tension produced in the nerves tends to pull the retina of the ocellus away from the lens. It was necessary to take precautions against desiccation, otherwise the nerves dried up within about 3 min. The ordinary type of moist chamber could not be used in conjunction with the micromanipulator, and the frequent use of Ringer was inconvenient. Good preservation of the nerve was first obtained by raising the humidity of the laboratory to a high value, but later it was found possible to immerse the whole preparation in medicinal paraffin saturated with oxygenated Ringer. The nerve would live for at least an hour under these conditions.

The preparation was mounted on a paraffin block beneath a binocular microscope and fine hook electrodes used to make contact with the tissues. Fig. 2A shows a typical preparation and response. When the light was switched *on*, the electrode nearer the eye became positive to the one nearer the cut end of the nerve; when the light was switched *off*, this potential disappeared after an 'overswing' of varying size. As the nerve near the cut will be depolarized, it follows that light stimulation is accompanied by increased polarization at the ocellus, and darkness by decreased polarization.

These results were repeated (Fig. 2B) with one electrode on the cut nerve and one on any tissue in the head. The latter then acts as though in contact with the ocellus. The same form of response was also obtained with electrodes on the intact nerve, but this preparation is unsatisfactory owing to the uncertainty of the electrical connexions within the tissues.

The potential changes just described were never accompanied by nerve impulses, even when the condenser-coupled amplifier with a gain of 10^5 was in use. This may

have been due to some unsuspected damage in the preparation, or to the short distance between the electrodes; but the possibility remains that the ocellar nerve does no more than conduct an electrotonic spread to the brain. Further discussion of this will be deferred until after the next section.

(ii) The commissure response

The preparation was dissected from the ventral side, as before, and part of the brain laid bare. The circum-oesophageal commissures were cut close to the sub-oesophageal ganglion and one of them lifted free of tissue by means of the platinum electrodes. As usual the loud-speaker was in constant use and the results will be described as they were heard in this instrument.

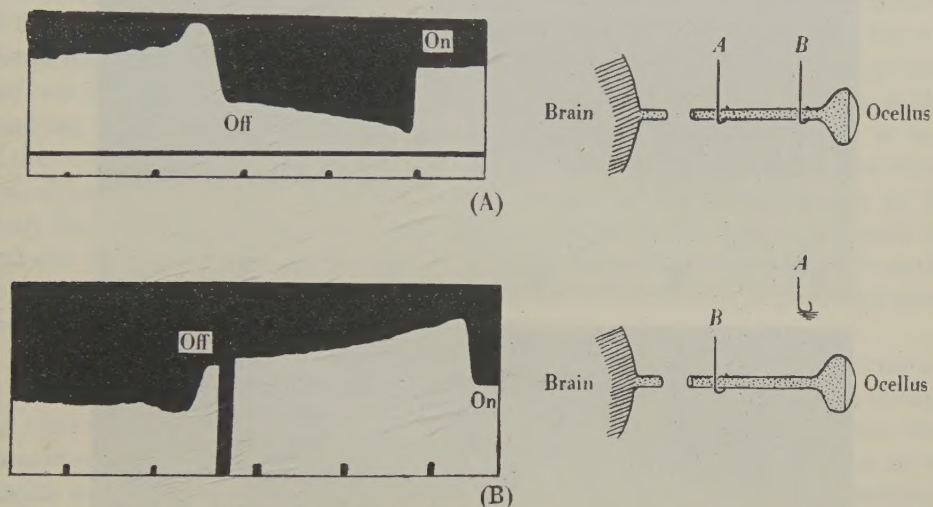


Fig. 2. Electrical response in the ocellar nerve when the ocellus is illuminated and then darkened. Explanation in text. Records read from right to left; upward deflection indicates *A* becoming positive to *B*; 2.0 cm. deflection equals 0.6 millivolts. Time marks every $\frac{1}{16}$ th sec.

Immediately after the dissection there was frequently a considerable amount of noise, but this died down after a short time and could be reduced by cutting the antennary nerves. The following phenomena were then clearly appreciated.

(a) *Optic and ocellar nerves intact.* The movement of an object in the visual field of the compound eye produced a loud response in the commissure. Fig. 3 A shows the response to a very slight movement. This response was only maintained while the object was actually in movement, and did not occur at all unless it was sufficiently well illuminated. If the object was in the visual field of only one of the compound eyes the response was heard only in the contra-lateral commissure.

When the visual field of the whole preparation was darkened a very characteristic response was heard in the commissure (Fig. 3 B). There was a sudden burst of impulses immediately the light was turned off, followed (sometimes after a short latent period) by a steady discharge which was maintained for several minutes before

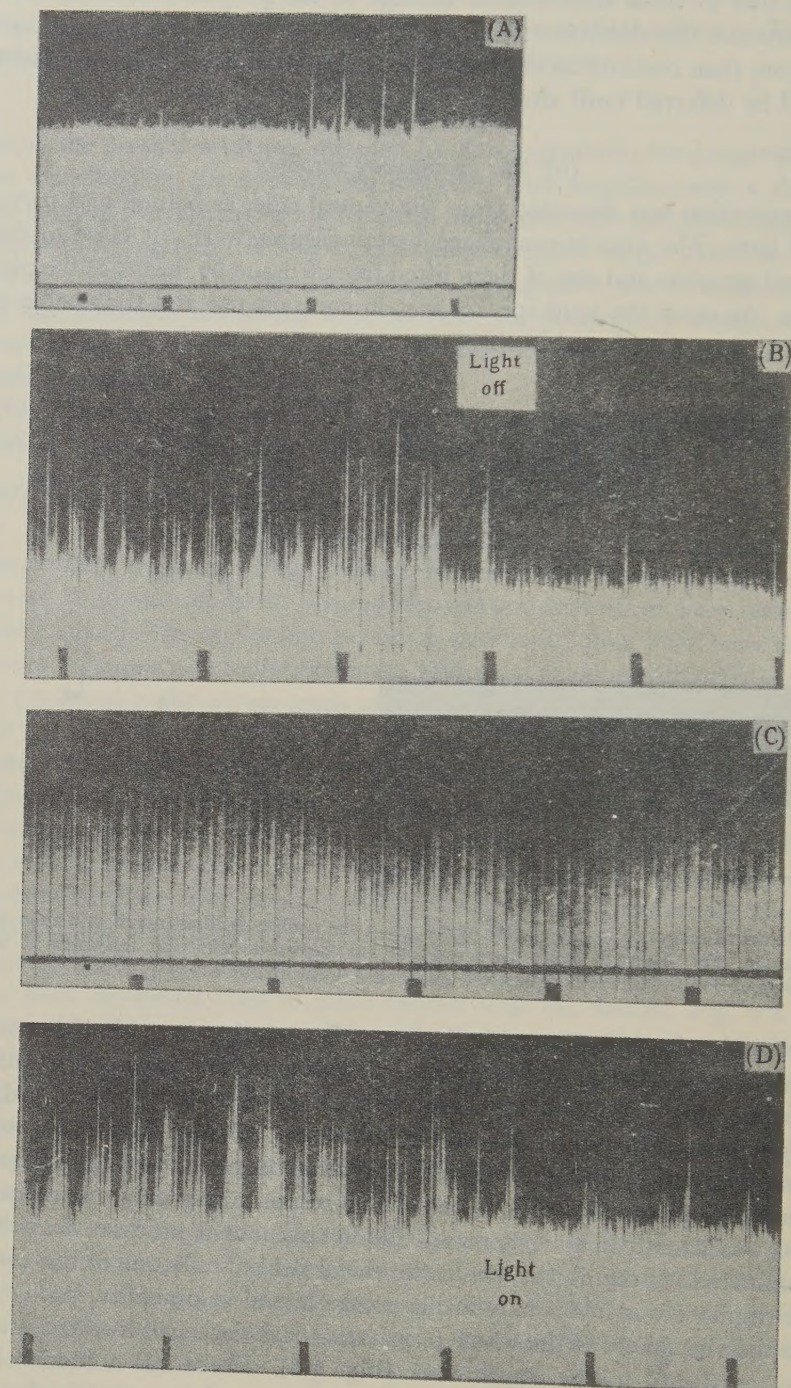


Fig. 3. Explanation in text. Records read from right to left. Time marks every $\frac{1}{10}$ th sec.

gradually dying away. If, however, the light was switched on before this time, the discharge immediately ceased.

The maintained discharge just described will be referred to as the 'darkness' effect, and the response to movement in the visual field as the 'movement' effect.

(b) *Optic lobes cut.* In such a preparation the movement effect was absent, while the darkness effect was as well marked as in the previous preparation.

(c) *Ocellar nerves cut.* The effect of cutting the ocellar nerves was very marked and surprising. It was the same whether the optic nerves were previously cut or not. Severing the frontal ocellar nerve had no effect beyond an 'injury' discharge of a few seconds, and possibly an increase in the darkness effect due to the intact lateral ocelli. When the latter were cut, however, there occurred, sometimes after a silent period of up to half a minute, a series of impulses of gradually increasing frequency, at first individually discernible but after a minute producing a low note. Fig 3 C shows this discharge after it has become fully developed. It was unaffected by light and was maintained for several minutes before gradually dying away. When it had done so, there was no darkness effect whether the optic lobes were intact or not. If they were intact, a darkening of the field produced a short burst of impulses which was almost certainly the 'movement' effect due to the change of intensity as the lamp filament cooled. The short burst was repeated when the lamp was turned on, and can be explained in the same way. Fig. 3 D shows this response, while at the beginning of the record can be seen all that remains of the discharge produced when the lateral nerves were cut.

The practical advantage of studying the ocelli by recording from the commissure is that, whereas the locust is the only available insect with ocellar nerves long enough for electrodes to be placed on them, there are probably a number of insects with commissures long enough for this purpose. It is thus possible to verify the above results with other species and to compare them with the responses to light obtained from insects not possessing ocelli. During this research the only other insect used was a dragonfly, which possessed ocelli, and in it the movement and darkness effects were both obtained, and also a note discharge when the ocellar nerves were cut.

DISCUSSION

We have found that when the ocellus is darkened it becomes depolarized and at the same time there is a burst of impulses in the commissure. The condition may be maintained for several minutes if the darkness is continued, but disappears immediately the ocellus is reilluminated. When this happens the ocellus becomes polarized and the impulses in the commissure abruptly cease. Now there is a considerable amount of evidence that the passage of impulses from a ganglion is preceded by a state of depolarization in the ganglion itself, while an increase in the polarity of the ganglion is accompanied by inhibition of impulses. Adrian (1931), working on the central nervous system of *Dytiscus*, placed an electrode on one of the ventral ganglia and another on the nerve cord. Before each burst of impulses he found that the ganglion became negative to the nerve, the record consisting of a slow

potential change with impulses superimposed. Barron & Matthews (1936) showed that when motor neurones in the frog's cord are reflexly stimulated an electrotonic spread may be detected in the ventral root, which they regard as an indication of depolarization within the cord. A motor volley may accompany this, depending on the extent of the depolarization. They also found that the application of negative potentials to the cord would produce an impulse volley in the ventral root.

These results suggest the following interpretation of the action of the ocellus. When the ocellus is darkened or the ocellar nerve cut, depolarization occurs in the ocellus, which spreads electrotonically down the very short nerve and depolarizes a ganglion within the brain, with the consequent discharge of impulses down the commissure. There are two independent pieces of evidence in support of this hypothesis which can most conveniently be described here.

First, it was found that when a potential of a few microvolts was applied between the ocellar nerve and the base of the brain, the nerve being negative, a continuous discharge similar to the darkness effect was recorded from the commissure. When the nerve was made positive this discharge immediately ceased.

Secondly, the ocellar nerves are histologically unusual. They contain very few fibres (four in the frontal and five in the laterals) of exceptionally large diameter— 25μ on the average—a condition also described in the bee by Kenyon (1896), and in the dragonfly and other insects by Cajal (1918). In view of the above experimental results it is interesting to note that Cajal describes the fibres from all three ocellar nerves, together with fibres from the optic lobes, forming a ganglion on the posterior face of the brain, just above the oesophagus.

It will require more evidence than is provided in the present paper finally to establish the existence of a nerve in which the usual nerve impulses are lacking. But whatever the mechanism of conduction in the nerve, the effect of varying light intensities on the ocellus is clear: darkening the ocellus (or cutting the ocellar nerve) results in discharges down the commissure, and these discharges are inhibited when the ocellus is illuminated and the nerve intact. It is known from behaviour experiments that darkening the ocellus does not by itself produce reflex activity, and therefore the results described above seem to give support to Wolsky's theory that the ocellus affects the general excitatory state of the nervous system. It might well be to an insect's advantage if certain reflexes were facilitated when the light intensity is reduced, as by a shadow; and it would be interesting to measure the threshold stimulus needed to evoke various reflexes involving the central nervous system when the ocellus is illuminated and when it is darkened.

SUMMARY

1. Little light is thrown on the function of the insect ocellus by blinding experiments or by a study of its distribution within the class.
2. The ocellus cannot, on optical grounds, receive an image, but can only be affected by changes in intensity of illumination.
3. The ocellar nerves are characterized by a small number of constituent fibres, and by the large diameter of the latter.

4. When the ocellus is darkened, the electrical response in the nerve consists of a decrease in potential near the ocellus, relative to the cut end of the nerve. No impulses were detected in the ocellar nerve.

5. When the ocellus is darkened, or one of the lateral ocellar nerves cut, there is a continuous discharge of nerve impulses in the commissures. This ceased as soon as the ocellus was reilluminated.

6. It is suggested that darkening the ocellus causes depolarization which spreads down the ocellar nerve and depolarizes a ganglion in the brain, thereby inducing the discharge of impulses down the commissures. It is pointed out that this might facilitate reflexes at lower centres, and so accelerate the animal's response to shadows.

This work was done in 1938-9 while I was receiving a Junior Research Award from the D.S.I.R., and was broken off on the outbreak of war. I wish to state my indebtedness to Dr A. D. Imms, F.R.S., for suggesting this work, and to Mr J. W. S. Pringle for putting his amplifier at my disposal, and for his advice during the research.

REFERENCES

- ADRIAN, E. D. (1931). *J. Physiol.* **72**, 132.
BARRON, D. H. & MATTHEWS, B. H. C. (1936). *J. Physiol.* **86**, 29P; **87**, 26P.
BOZLER, E. (1925). *Z. vergl. Physiol.* **3**, 145.
CAJAL, S. R. (1918). *Trab. Lab. Invest. biol. Univ. Madr.* **16**, 109.
GOTZE, G. (1927). *Zool. Jb. (Abt. Physiol.)*, **44**, 211.
HOMANN, H. (1924). *Z. vergl. Physiol.* **1**, 541.
KENYON, F. C. (1896). *J. comp. Neurol.* **6**, 133.
MÜLLER, E. (1931). *Z. vergl. Physiol.* **14**, 348.
PLATEAU, F. (1887-8). *Bull. Acad. roy. Belg.* **14-16**.
WOLSKY, A. (1930). *Z. vergl. Physiol.* **12**, 783.
WOLSKY, A. (1931). *Z. vergl. Physiol.* **14**, 385.
WOLSKY, A. (1933). *Biol. Rev.* **8**, 370.

LOCAL FACTORS AND REGENERATION IN CRUSTACEA

By A. E. NEEDHAM, *Department of Physiology, University of Manchester*

(Received 10 December 1946)

The role of normal innervation in the regeneration of crustacean appendages has been illustrated in previous papers (Needham, 1945, 1946); it may, perhaps, be termed the 'central' factor in regeneration. In the present paper 'local' factors, such as properties of the tissues near the site of amputation, have been studied. The results of earlier work, chiefly on *Amphibia*, *Planaria* and *Oligochaeta* (Korschelt, 1927, pp. 639-40), have suggested that the rate of regeneration varies with (1) the extent of tissue damage at the site of amputation and (2) the amount of tissue removed. These two factors have been studied in the present experiments on the regeneration of the 7th thoracic limb of *Asellus aquaticus* (L.).

METHOD

The general procedure was as in previous experiments (Needham, 1945, 1946). The effect of damaged tissue at the site of amputation was assessed by comparing the length of a normally regenerating limb at its first eclosion (its 'eclosion length', e.l.) with that of the partner limb regenerating in the presence of damaged tissue. This was arranged (i) by applying macerated tissue, chiefly muscle from the amputated limb itself ('ipseplasm'), to the stump immediately after amputation (series 7), and (ii) by light maceration of the stump *in situ*, through the autotomy membrane, immediately after amputation (series 8). The adhesion of the macerated tissue in series 7 was ensured by allowing it to dry after application, before returning the animal to water.

The distal aspects of the intersegmental joints were selected as suitable points for amputation in order to measure the relationship between regeneration rate and the fraction of the limb removed. It was hoped to compare, in a series of individuals, the regeneration of a distal fraction of n segments with one of $n+1$ segments in the partner limb, where $n=2, 3, 4$ and 5 in four different series. However, the frequency of autotomy subsequent to amputation (p. 223) prevented the collection of sufficient data for this direct comparison, and the results analysed below (pp. 221-4) were obtained by pooling all data for each value of n and comparing mean regeneration rates. The pooled data for $n=2, 3, 4, 5$ and 6 will be referred to as series 9, 10, 11, 12 and 13 respectively. On account of the shortness of the dactyl it was considered improbable that data for $n=1$ would be reliable. Measurements were taken, as before, at eclosion.

Series 9-13 were designed to measure the average regeneration rate for regeneration initiated in the different segments of the limb. In addition, regeneration from a point of amputation immediately distal to the intersegment was compared with regeneration from a point in the distal half of the segment, in order to detect

variations in 'regeneration-initiating potency' within the segment. This experiment was restricted to the carpus (series 14). As in series 9-13 insufficient complete pairs of results were obtained, and the analysis was applied to all 'basal' and all 'distal' regenerates.

RESULTS

Macerated ipseplasm (p. 220) applied to the stump of an amputated limb exercised an insignificant effect on regeneration rate (Table 1, series 7). Maceration of the stump itself caused (Table 1) a marked retardation of regeneration ($t=3.18$, $P<0.01$). Preliminary experiments involving the actual implantation of macerated tissue into the stump also gave evidence of retardation of regeneration. Macerated ipseplasm would appear to provide no stimulus to regeneration in this limb; on the contrary, maceration of tissues immediately proximal to the autotomy membrane caused a marked loss in regenerative power. These tissues would seem to possess a specifically high regenerative power, a conclusion in agreement with the observations of Przibram (1909, p. 116) that amputation at a point more proximal than the autotomy plane leads to a marked loss in power. This effect is so marked that statistical tests of significance seem unnecessary.

Table 1. *Asellus aquaticus*. Effect of macerated ipseplasm on rate of regeneration

All measurements in mm. $\times 56$ in all tables.

Series 7. Macerated ipseplasm applied to stump after amputation.

Series 8. Tissues of stump macerated immediately after amputation.

Series no.	No. of individuals	Mean abdomen width (mm. $\times 56$)	Mean experimental limb length	Mean control limb length	Mean difference between control and experimental limb length
7	39	110.1	139.4	142.4	3.05
8	38	126.9	113.4	138.7	25.32

The indication of high regenerative potency in these tissues gives added significance to experiments on regeneration from points distal to the autotomy plane (series 9-13). The mean e.l. (p. 220) of the regenerated portion of the limb decreased progressively with the distal movement of the point of amputation. The decrease between $n=6$ and $n=5$ was much greater than elsewhere along the limb, and regeneration is undoubtedly much more rapid from the autotomy plane than from any other point proximally or distally to it.

Although the e.l. of the whole fraction regenerated decreased progressively with the distal movement of the point of amputation, the mean e.l. per segment regenerated increased progressively in the same order, except that, even on this criterion, regeneration potency is greatest at the autotomy plane ($n=6$, series 13, Table 2). The mean e.l. per segment was calculated by dividing the total e.l. of the fraction regenerated by $n-\frac{1}{2}$, using the approximation that segments ischium (*I*), merus (*M*), carpus (*C*), and propus (*P*) are equal in length and the dactyl (*D*) approximately a half of this.

The mean e.l. of individual segments showed the same progressive increase from $n = 5$ to $n = 2$ (Table 2). In order to obtain a reliable estimate of relative regeneration rates the body size and time interval between amputation and eclosion ('eclosion time', e.t.) should be taken into account. The effect of variations in these two factors was eliminated by the approximate method of dividing the mean e.l. of any dimension by the product of the mean body size and the mean e.t., in days. This quotient may

Table 2. *Asellus aquaticus*. Regeneration of n distal segments of thoracic limb, where $n = 2, 3, 4, 5$ and 6 respectively in series 9, 10, 11, 12 and 13

No. of segments amputated (n) ...	6	5	4	3	2
Series no. ...	13	12	11	10	9
No. of records ...	99	37	31	99	45
Frequency of autotomy (%) following amputation ...	—	68	21	14	2
Mean e.t. (in days) ...	16.0	15.2	16.7	17.5	17.5
Mean abdomen width (mm. $\times$ 56) ...	118.4	126.1	117.6	128.1	124.1
Mean e.l. of limb segments:					
Basipreischium	*32.08	56.28	51.44	61.69	54.50
Ischium	23.69	*14.51	40.25	51.94	45.19
Merus	21.97	16.12	*17.46	44.83	41.20
Carpus	22.22	13.97	15.76	*22.95	41.61
Propus	21.76	12.71	14.28	20.12	*23.04
Dactyl	10.15	6.84	7.96	9.57	9.61
Mean e.l. of all segments regenerated	131.7	63.83	55.46	52.64	32.64
Mean e.l. per segment regenerated	23.94	14.18	15.84	21.06	21.76
S.d.m.i. of regenerated segments:					
Basipreischium	0.0169	—	—	—	—
Ischium	0.0125	0.0076	—	—	—
Merus	0.0116	0.0084	0.0089	—	—
Carpus	0.0117	0.0077	0.0080	0.0102	—
Propus	0.0114	0.0066	0.0073	0.0090	0.0106
Dactyl	0.0054	0.0036	0.0041	0.0043	0.0044
S.d.m.i. using the basipreischium as a measure of body size:					
Ischium	—	0.0170	—	—	—
Merus	—	0.0189	0.0203	—	—
Carpus	—	0.0164	0.0184	0.0212	—
Propus	—	0.0149	0.0166	0.0186	0.0242
Dactyl	—	0.0080	0.0093	0.0089	0.0101
M.s.d.i. of propus, using basipreischium as a measure of body size	—	0.0174	0.0178	0.0203	0.0248

The asterisk marks the point of amputation.

be termed the specific daily mean increment (s.d.m.i.) in contradistinction to the mean specific daily increment (m.s.d.i.) obtained by averaging the specific daily increment (s.d.i.) for each individual record of a particular dimension. This latter more accurate measure of regeneration rate was used for specific statistical tests of significance, but the former less laborious method was applied in general. The trends observed in the table of e.l. were preserved in that of the s.d.i. (Table 2); indeed, irregularities in the general trends were reduced, and there can be little doubt of the reality of these trends. The larger differences in s.d.i. were shown to

be statistically significant: the difference in m.s.d.i. of the propus between the series $n=2$ and series $n=3$, 4 and 5 respectively gave values of $t=2.92$, 4.13 and 4.02 for which the probability is less than 0.01 . Both abdomen width and length of the persisting basipreischium were used as a measure of body size.

The results of series 9-13 show another feature of interest. When regeneration rates of individual segments of a fractional regenerate are compared with those of corresponding segments of the whole regenerate ($n=6$) it is seen that the difference is most marked for the most distal segments of the fraction and decreases proximally, suggesting a centre of relatively high regeneration rate at the proximal end of the regenerate. This was observed also in the regenerating whole limb (Needham, 1943, p. 57 and Text-fig. 7). It is clear that the relatively higher rate of regeneration of a particular segment (e.g. propus) or of the average rate per segment when n is small must be due, to some extent, to the progressive elimination of the lower end of this proximo-distal gradient in regeneration rate, as the site of amputation is moved distally along the limb. There would, however, appear to be a genuine progressive increase in 'regeneration-initiating potency' between series $n=5$ and $n=2$, as shown by comparing the values, in the four series, of e.l. or of s.d.i., for the most proximal segment of the fractions regenerated (assuming that I , M , C and P are normally approximately equal in length (p. 221). Similar comparisons for succeeding segments, in order distalwards, show the same trend (Table 2). Moreover, the data for the whole regenerate (series $n=6$) indicate that a relatively steep proximo-distal gradient in segmental regeneration rates may itself be an expression of low potency.

The dactyl probably causes a slight irregularity in the proximo-distal gradient. A more interesting anomaly is seen in the basal segments, I and M , of the fraction $n=5$ (Table 2). In this series the centre of highest regeneration rate would appear to be located in the segment M rather than in I , the basal segment of the fraction. The mean difference between I and M is statistically significant ($t=3.44$) if two individuals, which between them contribute half of the total variance, are discarded. (Note. In one of these two individuals the I was abnormally long on account of a persisting proximal fraction of the segment and in the other, by contrast, that segment was completely absent. The complete absence of a segment is a rare abnormality of regeneration of these appendages, and this example may indicate an inherent poverty of 'regeneration-realization' potency in the segment I . Realization potency is to be distinguished from regeneration-initiating power which also, as judged by regeneration rate per segment, is lowest in this segment (pp. 221-3).)

The frequency of autotomy following amputation decreased progressively with the distal movement of the point of amputation (Table 2).

Within a particular segment tissues near the base would appear to possess a higher regeneration-initiating potency than more distal tissues. The difference between the mean e.l. of segments $P+D$ for basal and distal carpal groups (p. 220) was not statistically significant, but, owing to variable regression of tissue following amputation, the site of regeneration in each group showed some variation. In the

'basal' group regression frequently affected the merus. The data for each group were therefore divided into three subgroups according to the site of initiation of regeneration; this was determined in the 'distal' group by the ratio C/M (i.e. the ratio of the length of persisting fraction of the carpus to that of the intact merus—a measure of the fractional length of the carpus), and in the basal group by the ratio M/I , a measure of the extent to which the merus was affected by tissue regression after amputation. The subgroup with the highest ratio of M/I , virtually unity (Table 3), contained the examples of 'true' basally initiated regeneration in the carpus. The m.s.d.i. of segments $P+D$ for this subgroup differed significantly ($t=4.03$) from that for the subgroup representing regeneration from the middle ($C/M=0.51$) of the carpus. Regeneration-initiating potency would appear to increase again towards the distal end of the segment ($C/M=0.65, 0.79$).

Table 3. *Asellus aquaticus*. Comparison of regeneration rates for regenerates initiated at different points in the segment

Basal group			Distal group		
No. of records	Ratio M/I	M.s.d.i. of segments $P+D$	No. of records	Ratio C/M	M.s.d.i. of segments $P+D$
14	0.80	0.026	9	0.52	0.015
14	0.95	0.026	9	0.67	0.025
15	1.01	0.031	9	0.81	0.023

The main results of series 9–14 may be summarized as follows: (1) Regeneration-initiating power is maximal immediately proximal to the autotomy plane. (2) Subsidiary centres of high potency are located immediately distal to each intersegment. (3) As measured by the total length regenerated in unit time, the potency of these subsidiary centres decreases progressively in the proximo-distal direction, whereas, measured by the length per segment regenerated, the potency decreases equally regularly in the opposite direction (excepting regeneration from the autotomy plane, which, on any criterion, is more rapid than regeneration initiated at any other point of the limb).

DISCUSSION

There would seem to be little evidence that, in these limbs, regeneration is accelerated by a substance of the nature of a 'wound hormone', liberated from damaged tissues at the site of amputation. The autotomy mechanism no doubt normally ensures a minimum of damaged tissue at autotomy, and, moreover, this is separated from the regenerating tissue by the autotomy membrane, and it might be suggested that a sensitivity to possible wound stimulants has in consequence no adaptive value in these limbs. Nevertheless, regeneration from a point of amputation distal to the autotomy plane is not uncommon in nature, and is usually followed by evident and sometimes considerable destruction of tissue, yet regeneration is invariably slower than regeneration from the autotomy plane. Moreover, regeneration is slower from the middle of the carpus (p. 223) than from the base, where the amount of damaged tissue, as measured by tissue regression following amputation, is probably less.

The evidence for a regeneration-stimulating wound hormone, well established for plants (Haberlandt, 1922), is not strong for animals. Apart from the conclusions of Bier (1917), working on human regeneration, there have been few indications (Needham, 1941, p. 81) of such a factor. The familiar phenomenon of supernumerary regeneration or 'superregeneration' (Korschelt, 1927, pp. 413-72) is commonly believed to be due to such a factor, though, from the evidence, a mechanical morphogenetic cause appears at least as possible as a chemical one, and, moreover, the eventually excessive regeneration does not necessarily imply accelerated regeneration.

Far from stimulating regeneration in these limbs, maceration of the tissues immediately proximal to the autotomy membrane appears to cause marked retardation. The equally marked retardation which results from elimination of these tissues by amputation proximally to the autotomy plane (p. 221) strongly supports the suggestion that they possess an inherent maximum in regeneration-initiating potency.

It seems probable that relative maxima of regeneration-initiating potency may be located at those points where the incidence of regeneration initiation is relatively high. This is clearly true of the maximum near the autotomy plane, and it seems probable that regression of tissue, subsequent to amputation at points distal to this plane, may lead to a relatively high incidence of initiation from the intersegmental regions, where the narrowing of the lumen of the limb facilitates the arrest of regression. Again, if the regeneration rate per segment regenerated (pp. 221-3) may be taken as a measure of regeneration-initiating potency, this is also related to the normal incidence of regeneration at the different intersegments (i.e. inversely related to the incidence of autotomy following amputation at the various intersegments (p. 223).

The total regeneration rate of a fractional regenerate, as opposed to its segmental regeneration rate, varies with the amount of the limb removed (p. 221), supporting the classical view (p. 220), though, as shown above (p. 221), the relation is by no means linear.

Although the variations in segmental regeneration rates have been stressed in this paper, their approximate equality is perhaps remarkable. However many segments require replacement each acquires approximately the same length by the time of eclosion, suggesting that once the rudiment of each segment has been determined its further growth is ensured, at a rate which is inherently the same in all segmental rudiments, apart from such environmental accidents as its relative position in the limb, and the regeneration-initiating potency of the tissues at the site of origin of regeneration.

The proximo-distal gradient in regeneration rate along a fractional or complete regenerate (p. 223) is probably related to the familiar basifugal mode of regeneration (Korschelt, 1927, pp. 328-30). It is undoubtedly most marked in relatively small regenerates, due to early eclosion or to retarded regeneration.

SUMMARY

1. No evidence has been obtained of a wound hormone which stimulates regeneration of the thoracic limb of *Asellus aquaticus*.
2. On the contrary, damage to the tissues immediately proximal to the autotomy plane significantly retards regeneration, which depends considerably on intrinsic regeneration-initiating potencies in the intact tissues at the site of amputation.
3. This potency is greatest in tissues immediately proximal to the autotomy membrane, while subsidiary regions of relatively high potency are located immediately distal to each intersegment.
4. The absolute regeneration rate of amputated fractions of this limb increases with the extent of the fraction, according to classical views.
5. The increase is not linear, so that the regeneration rate per segment regenerated, though highest for a complete (6-segment) regenerate, originating at the autotomy plane, is lowest for a 5-segment regenerate, and increases progressively for 4, 3 and 2-segment regenerates.
6. The incidence of autotomy following amputation increases progressively with the disto-proximal movement of the point of amputation.
7. These features probably have adaptive significance.
8. Within a regenerating limb or fraction there is a proximo-distal gradient in regeneration rate, most marked in early stages of regeneration.

REFERENCES

- BIER, A. (1917). *Dtsch. med. Wschr.* **43**, 833.
HABERLANDT, G. (1922). *Biol. Zbl.* **42**, 145.
KORSCHOLT, E. (1927). *Regeneration und Transplantation*, I. Berlin.
NEEDHAM, A. E. (1941). *Proc. Zool. Soc. Lond. A*, **111**, 59.
NEEDHAM, A. E. (1943). *Proc. Zool. Soc. Lond. A*, **113**, 44.
NEEDHAM, A. E. (1945). *J. Exp. Biol.* **21**, 144.
NEEDHAM, A. E. (1946). *J. Exp. Biol.* **22**, 107.
PRZIBRAM, H. (1909). *Experimental Zoologie*, **2**, Regeneration. Leipzig.

STUDIES ON PLASTRON RESPIRATION

I. THE BIOLOGY OF *APHELOCHEIRUS* [HEMIPTERA, APHELOCHEIRIDAE (NAUCORIDAE)] AND THE MECHANISM OF PLASTRON RETENTION

BY W. H. THORPE

Sub-Department of Entomology, Zoological Department, University of Cambridge

AND D. J. CRISP

Department of Colloid Science, University of Cambridge

(Received 9 November 1946)

(With Plates 6 and 7 and Twenty-eight Text-figures)

I. INTRODUCTION

The probability that certain aquatic insects are able to extract oxygen from solution in the water by means of the air bubbles which adhere to their bodies was first suggested by J. H. Comstock in 1887, but it was not until Ege studied the subject in 1918 that the process was at all understood. Previously to Ege's experiments opinion had wavered between the theory that either the air stores carried by aquatic insects were hydrostatic in function (Brocher), or that they were genuine stores of air on which the insect could draw for its oxygen requirements whilst submerged, most workers rightly holding a combination of the two. The work of Ege confirmed the suggestions of Comstock and of a number of subsequent observers that the air bubbles carried by such insects as Dytiscidae, Notonectidae and Corixidae, besides constituting an air store, can, in fact, be used as a kind of 'physical gill'. Thus in many beetles a certain part of the surface of the subelytral air store is exposed to the water by abdominal movement. In other beetles, such as the Hydrophilidae and in the corixid bugs, an air film is carried entangled in the body-surface hairs, this film connecting with the spiracles and so providing a means whereby oxygen from the water can diffuse into the tracheal system. Insects which have such an air film entangled in their pubescence of course appear silvery when submerged. This system, consisting of the hairs and the layer of entangled air, was termed by Brocher (1912*b*) the 'Plastron', a convenient term equivalent to *Lufthulle* of Schaufuss (1916). Gas-bubble respiration and plastron respiration in the original sense thus involve simply the transport of oxygen from the surrounding medium to the internal gaseous environment across an air-water interface formed by a thin film or bubble of gas held on the body surface by suitable hairs. Ege showed that some larger species of aquatic insects can only get enough oxygen by diffusion into their gas films or bubbles when they are quiescent, and that any considerable degree of activity involves frequent visits to the surface in order to renew the oxygen supply by means of pumping movements ventilating mechanically the air spaces and tracheal system. But in other smaller bubble-carrying insects, for instance, *Hyphydrus* and the smaller species of *Corixa*, the gill effect of their gas bubble or film supplies them with sufficient oxygen to render them practically independent of visits to the surface even

when active. In the quiescence of winter conditions gas-bubble respiration may be sufficient to supply all needs, even of relatively large insects, and even under the ice there are always bubbles available for occasional renewal. The great value of Ege's work, however, was that he showed clearly that insects which respire in this manner are subject to a serious danger if they stay below for too long a period without having access to the atmospheric air or to bubbles—even though the medium is saturated with oxygen at atmospheric partial pressure. He showed that the successful functioning of an air store as a gill depends on the fact that the 'invasion coefficient' of oxygen between water and air is more than three times as great as that of nitrogen.* Therefore, if the tension of oxygen in the water is higher than the partial pressure of oxygen in the air film there will be a tendency for equilibrium to be restored both by diffusion of oxygen into the bubble and by the diffusion of nitrogen out of it. This mechanism is probably of some value in almost all aquatic insects which have any exposed bubble surface, for Ege showed that a *Corixa* or *Notonecta* will, because of the oxygen invasion, benefit to the same extent as it would were the air volume which it carries about thirteen times what it actually is. In an oft-quoted experiment Ege showed that a *Notonecta* which lived 7 hr. in a quantity of water saturated with atmospheric air (access to the surface being denied) was only able to live 35 min. in water saturated with oxygen (provided that the animal was placed in an oxygen atmosphere for a while before the experiment began).

The accepted interpretation of this experiment is as follows. When the animal is in a large volume of water saturated with air, then the bubble will contain nitrogen as well as oxygen. As the oxygen in the bubble is used up by the animal the tension of oxygen in the bubble falls and the tension of nitrogen rises; oxygen therefore diffuses into the bubble and nitrogen diffuses out. In this way the animal is able to make use of the oxygen dissolved in the water. If the quantity of nitrogen in the bubble was constant this process could go on indefinitely, but since nitrogen diffuses out into the water the result is that the bubble becomes smaller and eventually water will enter the tracheal system. When the bubble contains pure oxygen and the animal is in water saturated with oxygen, there is no tension difference to cause oxygen to diffuse into the bubble. Under these circumstances the oxygen dissolved in the water is not available to the animal, which is therefore only able to survive until the oxygen originally present in the bubble is used up. In fact, in these smaller bubble-carrying insects in which this 'physical gill' principle plays a major part, it may well be that the periodical visits to the surface are required rather to renew the store of nitrogen than to obtain a fresh supply of oxygen.

There are certain insects, however, which appear to have surmounted this danger and are able to hold gas in such a manner that it cannot under any normal circum-

* The idea of an invasion coefficient is spurious, but since the gas must diffuse through a thin stationary layer of the medium at the interface, the fiction is a useful one in defining the permeability of this layer. The coefficients of diffusion of oxygen and nitrogen in water will be practically identical. The permeability of water to oxygen is about twice as great as that to nitrogen owing to the greater solubility. Ege's figure for the invasion coefficient of oxygen of three times that of nitrogen seems doubtful, but since the invasion coefficient is wholly dependent on velocity gradients at the interface, experimental differences in convection during determinations may account for this.

stances dissolve in the medium and be replaced by water. *It seems advisable henceforth to restrict the term plastron to the extremely thin gas layer held by these insects in contradistinction to the easily replaceable air films and bubbles ('air stores') of insects such as Hydrophilus*

This arrangement differs fundamentally from an air store in that the volume of gas in the plastron is negligible and, moreover, the interface is held in position by surface forces so that the volume of the plastron is sensibly constant. The plastron therefore cannot function as an air store; its volume does not decrease nor does its air require to be replaced at the surface at intervals as the gas is used up. Owing to the tenacity with which the gas film is held in such an organ, differences of pressure may exist between the plastron space and the surrounding medium; therefore functionally it resembles more nearly the closed tracheal gill than the air store—even though the latter may to a limited extent also act as a physical gill.

The whole subject of plastron respiration in this strict sense is one of considerable theoretical interest and hitherto by no means understood. There are, briefly speaking, three groups of insects which show this phenomenon, but for various reasons they have hitherto all proved difficult to work with. *Aphelocheirus* (Hemiptera—Heteroptera, Aphelocheiridae) has always been described as extremely rare in western Europe. The same is true of the chrysomelid (Donaciine) beetles of the genus *Haemonia* (*Macrolea*), which have the additional disadvantage, for reasons which will appear later, of being extremely sluggish. The remaining group, Elmidae (Coleoptera Diversicornia), has many species which are common enough, but their very small size and again their sluggish habits render them very unsatisfactory for most types of experiment.

Brocher (1912*b*) showed that *Elmis aeneus* was able to survive more than 50 days' submergence even without the presence of gas bubbles in the water, provided, of course, that the water contained sufficient oxygen; and some desultory observations by other workers suggest the same thing in the other two groups mentioned. As long as the oxygen tension in the water remains sufficiently high these insects then appear able to supply their needs from the oxygen diffusion via the gaseous plastron. This raises a number of interesting theoretical problems—How is the plastron held? By what means is the danger of losing nitrogen avoided? As pointed out above the minute size of the Elmidae renders them difficult to work with, and because of their sluggish habits and their tolerance of considerable periods of oxygen want, they show no immediate and characteristic attitude denoting asphyxia. *Haemonia*, although larger, has the same objections and appears, moreover, to be excessively rare. *Aphelocheirus* is large and active and is therefore in many ways the ideal animal for experiments.

The senior author's interest in *Aphelocheirus* was first aroused by Szabo-Patay's (1924) account of the remarkable rosette-like structures, evidently the spiracles, which are found on the abdominal sterna of this insect. There are many similarities in respiratory adaptations between aquatic and parasitic insects, and Szabo-Patay's quite gratuitous and, indeed, untenable suggestion that these rosette-like structures were concerned with selective removal of oxygen from the water recalled other physical

assumptions implied in Maple's (1937) discussion of the so-called 'aeroscopic plate' on the egg stalk of the parasitic Chalcidae (cf. Thorpe, 1936). It appeared probable from Szabo-Patay's account as well as from the observations of some earlier workers that *Aphelocheirus* was an example of plastron respiration. Szabo-Patay saw that there was a fine hair pile over a considerable part of the body—such as might be expected to entangle a film of air for respiratory purposes (although this possibility did not apparently occur to him)—and it seemed probable that the rosette structures were in some way adaptations to this special mode of respiration. In effect, then, the opportunity to work on the subject of plastron respiration depended on the finding of a fairly copious supply of this apparently very rare hemipterous insect *Aphelocheirus*. A few occurrences extending over 70 years or more were on record for, in all, about ten localities in Great Britain and Ireland, but it was not until 1942 that Daniels and Ellis found *Aphelocheirus* abundantly in the River Wensum, near Norwich. On hearing of this record in 1943 one of us (W.H.T.) visited the locality and obtained some through the kindness of Mr E. A. Ellis of the Norwich Museum, to whom we have been indebted in many ways during the search for material for observation and experiment. About the same time Lt. E. S. Brown supplied valuable information about the occurrences of the insect in the River Cherwell, north of Oxford. For experimental purposes most of our material has been collected from one or other of these localities, the method being to don waders and walk slowly upstream through the shallow swift-running parts of the river, dragging a moderate-sized dredge, and disturbing the stones with one's feet. The animals can be transported to the laboratory in tins containing damp waterweed, under which conditions they will, if required, live satisfactorily for as long as 2 or 3 days until they can be transferred to a properly aerated aquarium.

The present paper is the first of a series of four or five. In those which follow it is proposed to deal first with other aspects of the biology of *Aphelocheirus* in relation to respiration and to describe a detailed study of the respiratory efficiency of this insect and the theoretical principles involved in plastron respiration. This is to be followed by an investigation into plastron respiration in the Coleoptera and a study of the structure and function of insect hair piles in general, with special reference to the evolution of gas-bubble and plastron respiration.

2. A BRIEF OUTLINE OF THE STRUCTURE AND BIOLOGY OF *APHELOCHEIRUS AESTIVALIS* FAB. FORMA *MONTANDONI* HORV.

Aphelocheirus is a large, tough, strongly flattened water bug, in appearance very unlike any other British aquatic insect. The form which occurs in this country is flightless, the hind wings being reduced to the merest vestiges hidden beneath the immovable semicircular forewings. A winged form, the status of which is obscure, is known from the Continent. *A. aestivalis* is normally characteristic of rivers or stretches of river with rapid current, well-oxygenated water, and a bottom of stones and small boulders on gravelly sand. It is a rapid swimmer, particularly at dusk, and if adults are kept in aquaria and observed at night they will be found to be highly active between 10 and 11.30 p.m., and occasionally later in the night or early in the

morning before dawn. At such times they are found to swim close to the surface or clamber actively among the weeds. This activity is not the result of bad aeration of the aquarium water due to cessation of photosynthesis by the aquatic plants, but occurs equally in aquarium tanks supplied with a bubbler. During the daytime the bugs spend a large part of the time under stones, often just submerged in the sand. They do not appear to be associated with any particular kind of plant, although probably the presence of *Potamogeton pectinatus* is as good an indication as any of a suitable environment for them. They are carnivorous in habit and feed by probing with the long proboscis under stones and among crevices of rocks. In this way they obtain chironomid larvae and the larvae of caddis flies, e.g. *Hydropsyche* (Sirotinina, 1921) and the molluscs *Vivipara vivipara* (E. S. Brown in litt.), *Cyclas* and *Pisidium* (Larsen, 1924). Although *Aphelocheirus* is normally found in the type of environment indicated, it has also been recorded from a surprising variety of situations; Decksbach (1921), according to whom it occurs in many places in the Upper Volga, found it in shallow and stagnant puddles left by the subsidence of the main stream and states that in these collections of water the animal will persist for many months. Larsen (1932) has similar records. Beling (1926) describes it from many types of habitat in the River Bug in Poland, while Kulmatycki & Gabanski (1928) record that in the River Wierzyca larvae are able to resist successfully winter pollution caused by a sugar-beet factory, a pollution so great that it brought about a dominant fungus flora in the water. There are also records from brackish water near Kiel, as well as in Finland, Denmark and in France near the mouth of the Seine. It is known also from lakes, there being records from the English Lake District and from Lough Neigh, Ireland. A striking feature of the insect is the fact that while most frequently found in fairly shallow water, it has been recorded as going to a depth of 4 m. at Gudenaa in Denmark (Ussing, 1910), 5 m. in the Seine (Royer, 1913), and as much as 7 m. in the Volga (Decksbach, 1921, 1923). The eggs are laid mainly on stones and the life history is a long one, probably at least a year. All stages may be found at all times of the year. In Europe the species is apparently abundant in the Volga and some other Russian rivers, and is known from Sweden, Finland, Denmark, Poland, Germany, Austria, Hungary and France, but the general impression given by the records is that the insect gets progressively rarer towards its western limits. Szabo-Patay's paper, for instance, was written as a result of seeing a single live specimen taken from the Danube. The distribution of the animal in the British Isles is somewhat remarkable and is not fully understood. One would think that such a relatively large (1 cm. long) and unusual looking insect—it suggests a huge aquatic bed-bug—could not fail to be recorded in most places where it occurred; but in actual fact the recorded localities even now (Bedwell, 1945) number less than twenty widely scattered areas in England, from Sussex, Hants, Berkshire, Norwich and Oxfordshire in the south to Berwick-on-Tweed in the north, Shropshire and north Wales (Pearce, 1945) in the west, and one in Ireland.

In appearance the insect is of a dark mottled brownish grey colour above, and underneath it shows a delicate pale grey sheen. There is no visible air store. There is, of course, no proper subelytral air space, but a little air is found under the

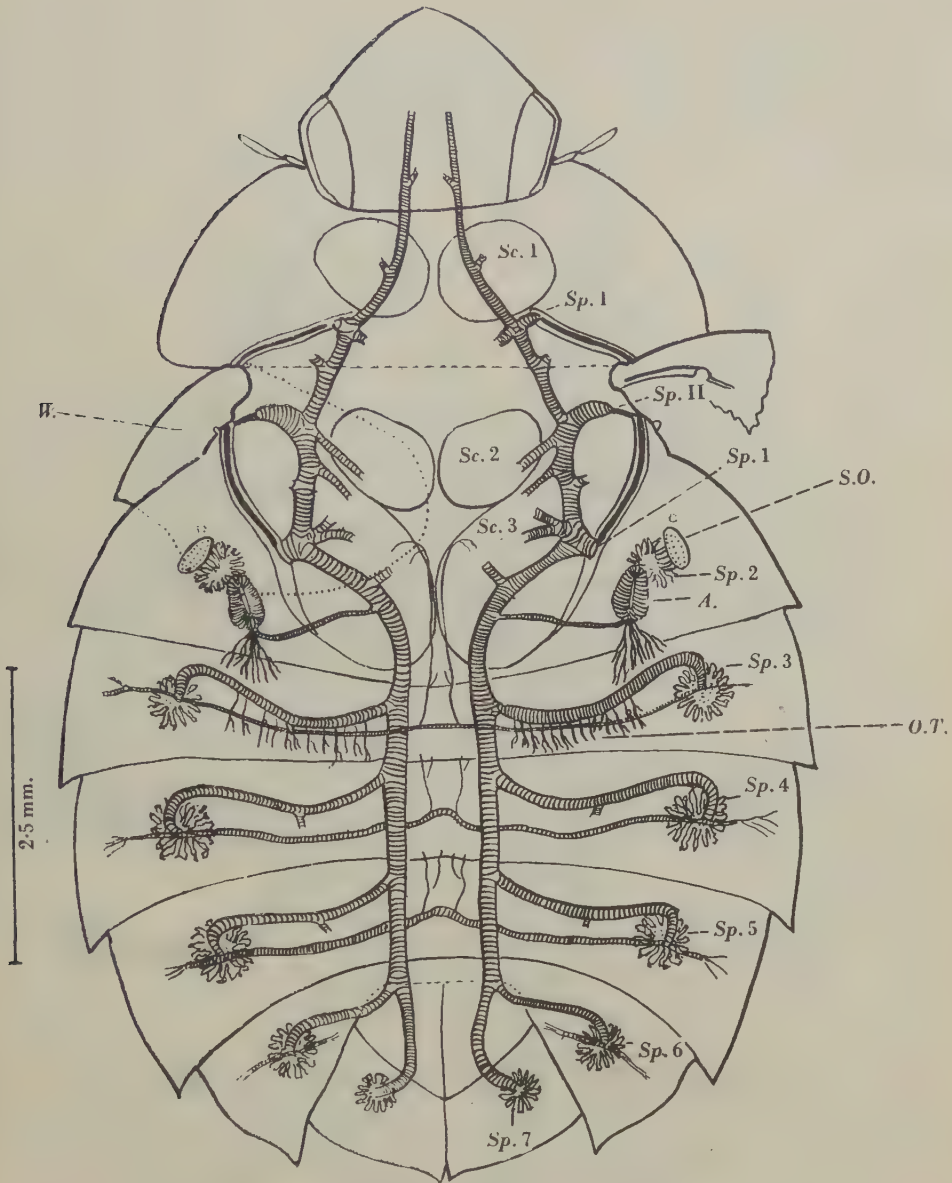
rudimentary forewings. Since there is no bulk of air or air bubble carried the insect is heavier than water, and so, unlike many other aquatic insects, does not immediately bob up to the surface when it ceases swimming, but sinks to the bottom. Ussing (1910) pointed out that *Aphelocheirus* never normally comes to break the surface though it may swim about just underneath. It does not show respiratory movement of the abdomen or thorax.

Aphelocheirus will live and breed in aquaria, provided they are well aerated with a bubbler. We have also kept them in a special running water aquarium, but it is doubtful whether this has any real advantages over the simpler type. The animals will even live well for a considerable time in shallow dishes without any special means of aeration. As will be anticipated from the above-mentioned record of their survival of heavy pollution, they will survive for a long time in water containing much organic matter, provided they are not short of oxygen. If, however, the oxygen content falls the insects tend to swim more vigorously near the surface, often upside down, and may under such conditions try to climb out. If that fails they become sluggish and fall to the bottom, the hind end of the body then being raised slightly and the hind legs stretched out in a characteristic manner; these are the first signs of asphyxia. If oxygen want persists they then topple over on to their backs, giving spasmodic kicks—the second stage of asphyxia. Finally, they lie quiescent on their backs with their hind legs extended and crossed—third stage of asphyxia. If not left too long without aeration they will recover rapidly when transferred to aerated water. The nymphs are similar in general appearance but they show no ventral sheen, the skin is thinner and the whole insect somewhat transparent. They are more sluggish than the adult, but are capable of vigorous swimming. For a full description of the nymphal stages see Larsen (1927).

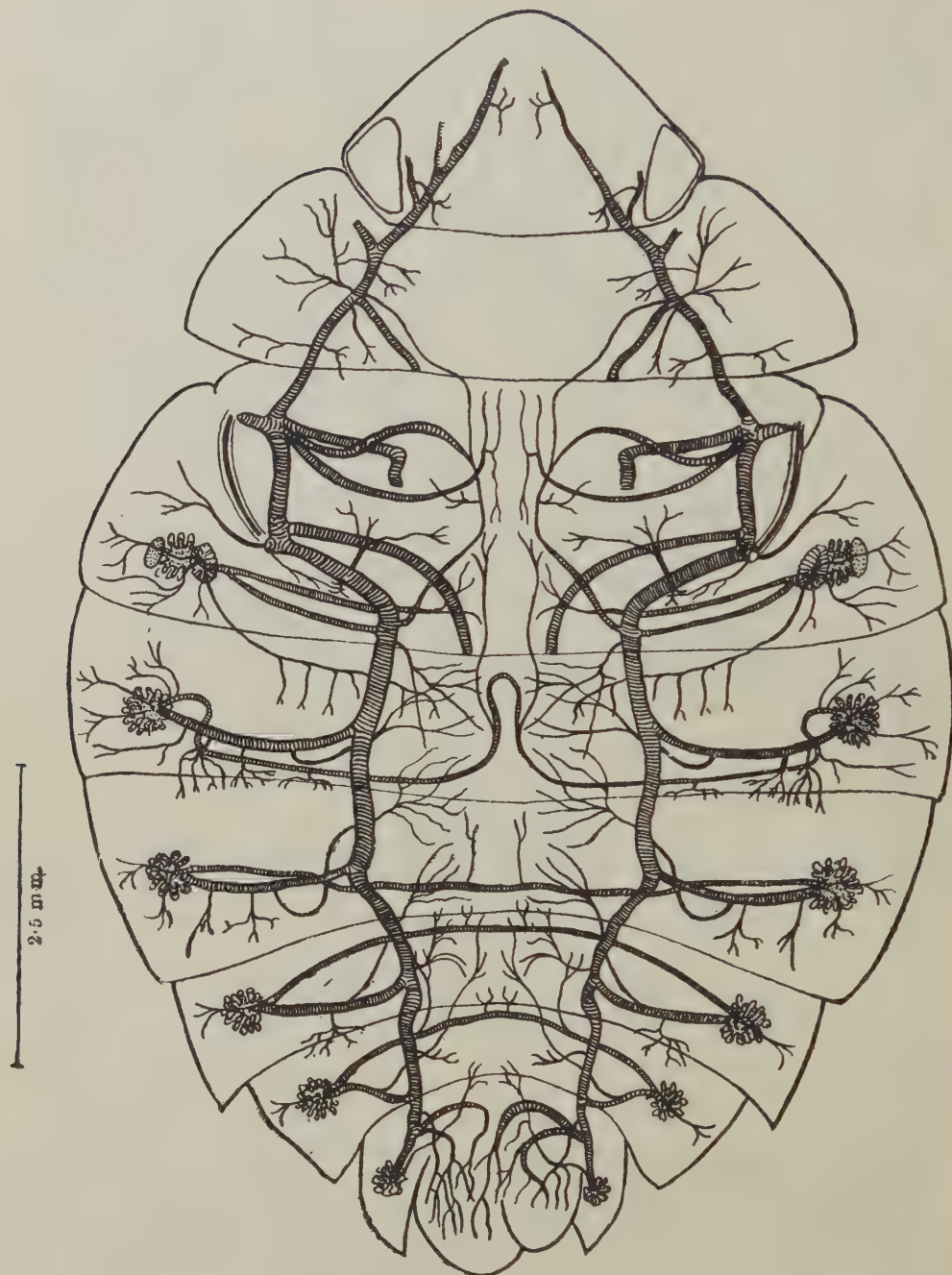
3. RESPIRATORY SYSTEM OF ADULT *APHELOCHEIRUS*

(a) *Tracheal system and spiracles*

Szabo-Patay, in his 1924 paper, gives a good account of the tracheal system of adult female *Aphelocheirus*, and Text-fig. 1 is largely based on his paper, but has been corrected on some points in accordance with Larsen's description (1924) and our own observations. Text-fig. 2 shows the tracheal system of the male in much greater detail. As will be seen from the figures, the tracheal system consists of two main tracheal trunks extending almost the whole length of the body. There are nine pairs of spiracles in all, two thoracic and seven abdominal, those belonging to the eighth abdominal segment having disappeared as in some other families of the Hemiptera (e.g. Cimicidae, Aphidae; see Weber, 1930, p. 272). The spiracles are of two entirely different types. The two pairs of thoracic spiracles and the first abdominal spiracles open into deep grooves running roughly along the sides of the much-flattened thoracic segments. The anterior thoracic spiracle is situated ventrolaterally at the junction between the pro- and mesothorax. From it a groove runs outward and upward meeting a small channel which connects with the second thoracic spiracle near the point of the attachment of the much-reduced mesothoracic wing, thereby connecting with the small air space between that wing and the dorsal surface of the

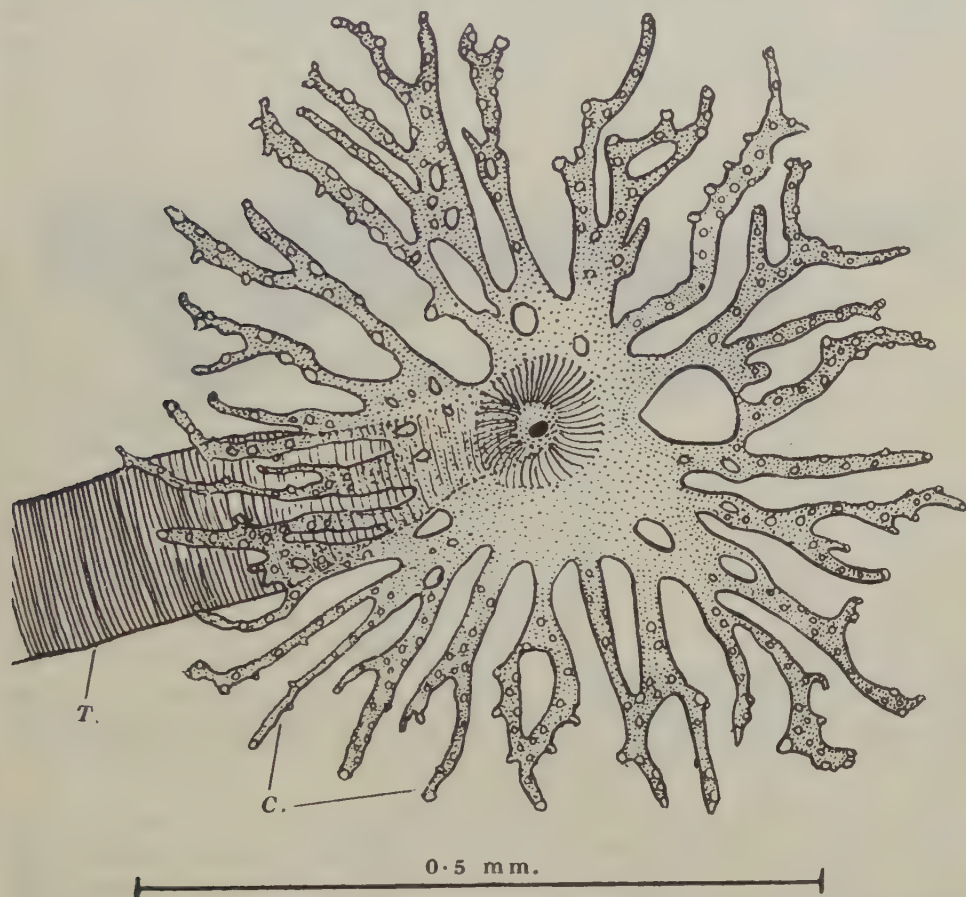


Text-fig. 1. Respiratory system of female *Aphelocheirus aestivalis* dissected from the dorsal surface. (Modified from Szabo-Patay.) Only the main tracheal trunks are shown. *Sp. I*, *Sp. II* = thoracic spiracles. *Sp. I* = first abdominal spiracle. *Sp. 2-7* = 'rosettes', being the abdominal spiracles. *A.* = air sac. *S.O.* = sense organ on sternum of second abdominal segment. *Sc. 1, 2 and 3* = pro-, meso- and metathoracic subcoxal plates. *O.T.* = tracheae supplying ovaries. *W.* = rudimentary mesothoracic wings. (Dotted line shows position of left wing when in normal position. The right wing has been raised from the position in which it is fixed in the living insect, only the base of it being shown.)



Text-fig. 2. Respiratory system of male in detail.

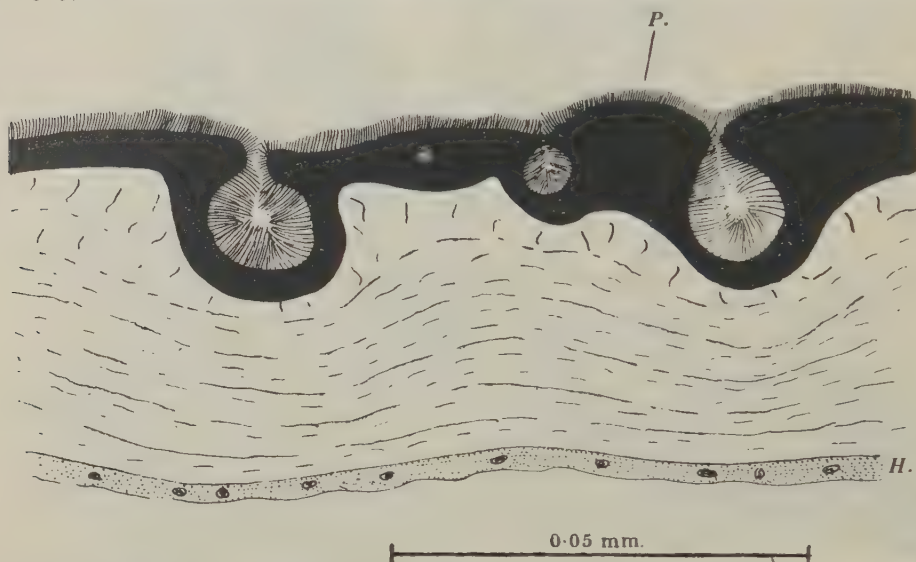
metathorax and the second abdominal segment (i.e. the first abdominal segment to be well developed). The groove then runs back along behind the margin of the meta-thoracic subcoxal plate to meet the first abdominal spiracle at the point indicated. The groove becomes shallower and quickly fades out posterior to this point. These three spiracles each have a single very minute opening, and the groove with which they connect is closely packed with a thick-set pile of minute hairs. The structure of



Text-fig. 3. Spiracular 'rosette' of third abdominal segment. *C.* = channels in exocuticle forming the arms of the rosette. The small circles and bud-like projections on the arms are the minute openings of the spiracle. The central spiracular scar is shown as a black dot. *T.* = trachea.

these spiracles has been described by Larsen (1924), but his account appears incorrect in certain respects. As will be seen from Text-fig. 6, the mouth of the trachea is protected by a screen of long cuticular filaments themselves beset with fine hairs. This mass of hairs may appear in section, under certain conditions, as a thick plate perforated with holes, and this no doubt explains the discrepancy between Larsen's paper and our own observations. No closing apparatus appears to be present. The six abdominal spiracles situated on segments 2-7 inclusive are of an entirely different

structure. They show ventrally as pale pinkish circular scars or patches which are made up of a number of fine branches radiating from the centre, the whole giving the appearance of a rosette, which name Szabo-Patay applied to them ('Corpuscule en forme de rosette'). The appearance of a typical abdominal spiracle is shown in Text-fig. 3. The trachea which connects up with the main trunk can be seen underneath at about the middle point of the rosette. Sections reveal that each of the branching arms consists of a fine channel running through the substance of the exocuticle. As they pass from the centre outwards these channels become more superficial and open to the exterior by a number of very minute pores (see Text-figs. 3-5). There is a central scar near where the trachea is attached, but no opening

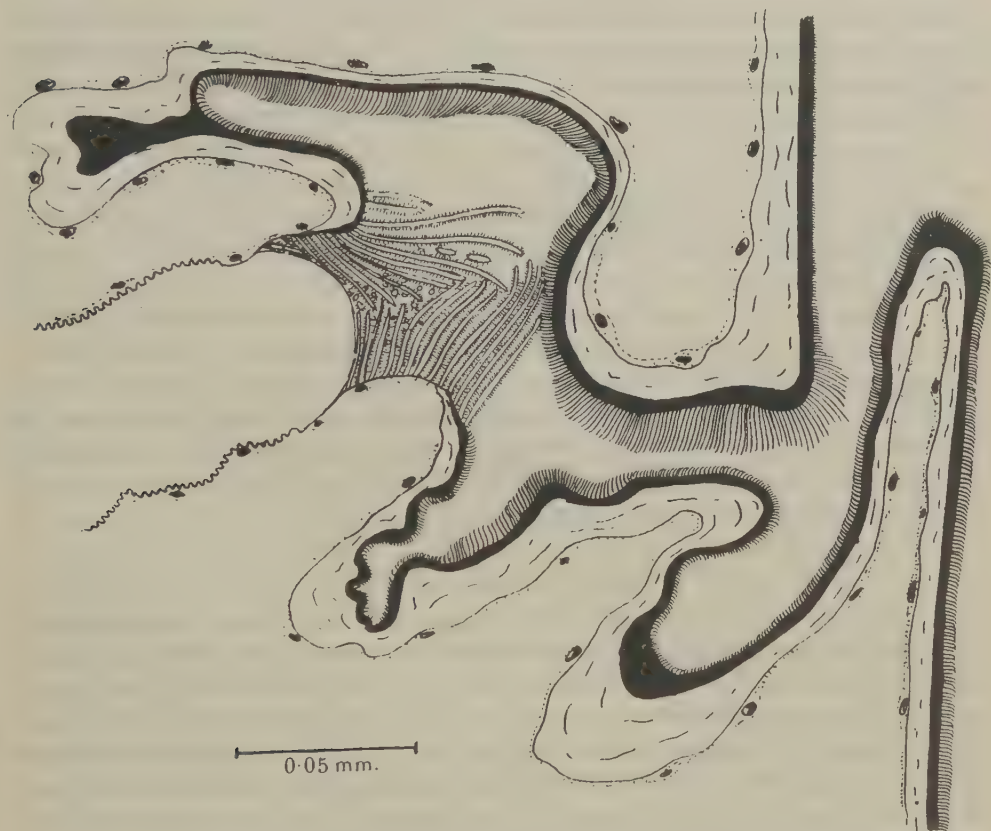


Text-fig. 4. Section through edge of rosette showing four of the channels in the exocuticle cut transversely. *H.* = hypodermis. *P.* = plastron hairs. Exocuticle black. Endocuticle showing lamellae. In the case of the two larger channels the section passes through one of the openings to the exterior. (From an unstained frozen section.)

is visible at this point. It will be seen from Text-fig. 4 that each of these grooves or channels is tightly filled with a hair pile which is, in fact, a continuation of the general hair pile which makes up the plastron of the ventral surface. At first inspection the spiracles give the impression of being closed, and it is not easy to make sure whether the tiny clear areas on the branches are in fact openings. That they are open is, however, clearly demonstrated by placing on the animal a minute drop of oil of cloves which has been heavily stained with Sudan black B. If watched under the binocular microscope the oil can be seen spreading along the surface and gradually filling the rosettes, and with a little care it is easy to see that the rosette branches fill from the tips and not from the centre. If the spiracle is now dissected out and mounted the drops of stained oil can easily be seen in the rosette channels. By this method the tracheal system can be completely injected with oil. As would be expected, the oil spreads more slowly down the arms of the rosette than it does across the ventral



Text-fig. 5. Section through centre of rosette showing junction of trachea with the system of channels which are here larger than at the margins and which thus, with their layer of exocuticle, bulge deeply into the endocuticle. Note screen of long cuticular filaments, beset with fine hairs, which protects the mouth of the trachea. (From material fixed in Duboscq Brasil, sectioned in celloidin paraffin and stained Mann's methyl blue-eosin. Exocuticle pink to purple. Layers of endocuticle blue.)



Text-fig. 6. Section through first abdominal spiracle situated in the deep-groove separating the meta-thoracic subcoxal plate from the sternum of the second abdominal segment. Note the screen of long cuticular filaments, beset with fine hairs, which protects the mouth of the trachea. (Duboscq Brasil, celloidin-methyl blue-eosin.)

plastron hairs of the surface. Szabo-Patay describes an elaborate arrangement at the centre of the rosette consisting of a movable plate which he assumes is capable of performing a kind of pumping movement. On this he advances a remarkable theory of the function of the rosette which is, in effect, that oxygen is adsorbed on to the very great surface provided by the innumerable hairs, and that it is then removed as a result of the pumping action of the structure referred to and so rendered available for diffusion into the insect's tracheal system! We have been unable to confirm the existence of this pump either in stained sections or in whole unstained preparations. Study with the polarizing microscope fails to reveal any muscles which could be operating any pump. From this and other considerations which will appear later, we conclude that there is nothing in the structure of the abdominal spiracles to support Szabo-Patay's theory of the mode of respiration. All the peculiarities of spiracle structure seem easily explained on quite another basis, and, indeed, Szabo-Patay's hypothesis appears mechanically, physically and physiologically unreasonable.

It will be seen that the trachea running to the rosette of the second abdominal segment is somewhat thinner than those supplying the others, and that it discharges into a curiously shaped air sac the walls of which are folded and ribbed in a peculiar concertina-like manner. Its function will be discussed in the third paper of the series. The air sac opens directly into the rosette of the second abdominal segment, and that rosette is cut short by the presence on its outer edge of an oval plate, the lateral branches of the rosette running straight into it. This plate shows in the living insect as a brilliantly shining silvery patch. A moderately high power of the microscope reveals it to be a depression in the cuticle covered with long backwardly projecting hairs which entangle a relatively thick layer of air. It is, in fact, an organ of pressure sense of a remarkable type, the function of which will be discussed in a later paper. A conspicuous nerve runs to it from the composite ganglion which is situated in the region of the metathorax. The chief remaining features of the tracheal system can be seen from Text-figs. 1 and 2 and need not be described in detail. The only point worthy perhaps of note is the well-developed ventral commissures which connect the two rosettes of each of the abdominal segments 3-7 inclusive.

(b) *Cuticular hair pile (plastron hairs)*

Larsen (1924) was the first to show that hairs similar to those found in the spiracular branches, but much shorter, cover the whole ventral surface of the animal as well as a considerable part of the dorsal surface. Owing to the dark colour of the dorsal surface the hair pile there is very hard to see. Ventrally, however, it gives a curious grey iridescent glint or sheen to the whole surface, whether the animal be examined in air or in water. Older individuals tend to get the hair pile scratched and worn and consequently appear darker, or with irregular dark patches. When the animal is removed from the water and the surface dried, it is noteworthy that the surface is not again readily wettable with clean water. This again seems to depend somewhat on the age of the insect, and one with a rather dark ventral surface will wet more easily—at any rate in the regions of the intersegmental membrane. A newly emerged adult,

however, is strikingly hydrophobic, and water placed on the surface falls off as if the insect were covered with wax. An insect which has been thoroughly dried has no little difficulty in entering the water again, and does not usually manage to do so quickly unless it can pull itself down by getting hold of a piece of waterweed or some other convenient object. That the sheen of the insect is due to the existence of the hair pile containing air is very easily demonstrated. If it is touched with a drop of absolute alcohol or a solution of a wetting agent such as butyl alcohol or cetyl pyridinium chloride, the surface immediately darkens. This is evidently due to the wetting of the minute hairs and the consequent expulsion of the contained air. If an insect thus treated is rinsed and immersed in water without being allowed to dry, it remains dark in colour. If, on the other hand, it is allowed to dry thoroughly in air, the sheen reappears and the surface resumes its former unwettable condition. The extreme permanence of the gas layer of the plastron is indeed a very remarkable feature of *Aphelocheirus*. The plastron hairs will in many cases retain their gas layer almost indefinitely after death. We have had specimens which were collected in Norfolk in 1942, put aside in a corked tube containing a little muddy and highly polluted water, which showed their sheen almost undiminished two years later!

In the following section we show experimentally that the plastron in *Aphelocheirus* can retain a gas layer under all normal conditions and that it does, in fact, serve a respiratory function.

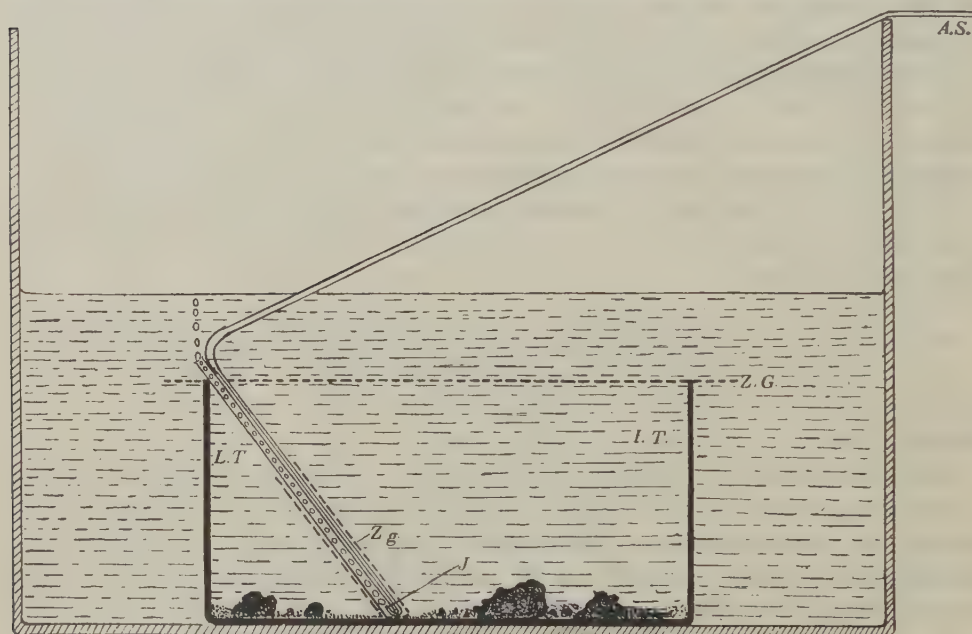
4. RESPIRATORY BEHAVIOUR OF *APHELOCHEIRUS AESTIVALIS* FAB. *FORMA MONTANDONI* UNDER DIFFERENT CONDITIONS

(a) *Adult*

(i) *Experiments on the prevention of surface access*

In considering the respiration of *Aphelocheirus* it is, of course, first necessary to make certain that it does not in fact need to come to the surface. To this end experiments on prevention of surface access were carried out, the animals being confined in a small glass tank (Text-fig. 7, *I.T.*), measuring $4 \times 4 \times 8$ in. They were provided with a suitable layer of gravel and stones at the bottom, but were without any aquatic plants. This tank was closed by a tightly fitting perforated zinc grating (*Z.G.*). The whole was then immersed in a larger glass tank containing water to the depth of $5\frac{1}{2}$ in. There was then $1\frac{1}{2}$ in. of water above the grating of the inner tank. A glass tube connected with the compressed air supply (*A.S.*) was led in through a hole in the zinc cover of the inner tank, this tube being bent round on itself at its tip (*J.*), which was inserted into another somewhat larger piece of glass tubing (*L.T.*). The two tubes were then fastened firmly together, and their lower ends protected by a perforated zinc guard (*Z.g.*) so that the bugs were unable to get up the larger tube or come into actual contact with air bubbles. When the air supply is turned on, bubbles then pass up the larger tube and out to the surface, drawing with them a steady stream of water, thus keeping the water in both tanks circulating and well aerated. A number of freshly collected female bugs were kept in this apparatus at a temperature of 18° C. the controls being in similar jars under normal aquarium conditions. It was found that the insects were able to live in an apparently healthy

condition for at least 12 days, the only difference between the experimental and control animals being that the former appear perhaps rather more restive than the latter. There was no change in the appearance of the plastron during the experiment. It can be safely assumed, of course, that if nitrogen loss had been occurring during this time the gaseous plastron would have completely disappeared. This would, in fact, be expected to occur in the first few minutes if not sooner, since the volume of gas in the plastron is so extremely minute. Brocher has described in *Haemonia* (1912 *a*) that minute air bubbles extruded from a micropipette will adhere to the plastron and be readily absorbed. We have not been able to observe anything of this kind in *Aphelocheirus*. Bubbles will sometimes cling for a little while to the marginal setae or to the leg bristles, but there was no adhesion or absorption as described by Brocher.



Text-fig. 7. Tank used for experiments on prevention of surface access. *I.T.* = inner glass tank. *A.S.* = air supply. *J.* = jet of air supply. *L.T.* = larger glass tube for ascending bubbles of air. *Z.G.* = perforated zinc grating covering inner tank. *Z.g.* = perforated zinc guard preventing insects coming into contact with air bubbles.

(ii) *Experiments on the retention of the gaseous plastron*

The next step was to see whether the insect is in danger of losing its gas layer even when in gas-free or oxygen-saturated water. It will be remembered that Ege has shown that a *Notonecta* placed in an oxygen atmosphere and then moved to oxygen-saturated water but denied access to the surface very rapidly loses its bubble; for the oxygen is absorbed, the carbon dioxide diffuses out and immediately dissolves, and in the absence of nitrogen no tension difference can be established between the bubble and the medium. Experiments of this kind with *Aphelocheirus* were quite conclusive. If insects are placed in a litre jar of water which has first been boiled

gently for an hour to remove all gas and then cooled and saturated with oxygen from a bubbler for a similar period, they remain perfectly normal, no change or abnormality in their behaviour being observable as compared with control insects. If the insects are placed in gas-free distilled water they respond immediately by great activity, swimming about at the surface trying to climb out of the tank, and sometimes succeeding. If prevented from climbing out they cling to the side, and if they accidentally drop down, immediately come to the surface again. After a few minutes their activity decreases and they fall to the bottom and soon pass through all the stages of asphyxia mentioned earlier. The final stage of asphyxia supervenes in about 35–40 min. at a temperature of 18° C., but bugs removed at any time up to about 2 hr. may show quite rapid recovery. More than about 2 hr. total deprivation of oxygen is sufficient to kill the animal, but during such an experiment there is no visible change in the plastron. To follow this matter further, the following experiments were performed.

A bug with a very good ventral sheen was decapitated, and the right half of the abdominal sterna of the last four segments was dissected and placed in 20 c.c. of freshly boiled distilled water under a 'Nujol'* seal. The left half of the same individual was placed in aerated distilled water under the same conditions. The experiment was continued for 144 hr. but no differences between the two were observable. A similar experiment was conducted with living insects. Freshly caught females with a very good ventral sheen were fastened on their backs on a cover-slide with a small quantity of Euparal. One specimen was placed in a conical flask containing 250 c.c. of freshly boiled distilled water run in and cooled under a 'Nujol' seal. Controls were kept in exactly similar conditions except that the water was well aerated. The experimental animal was thought to be dead in about 3 hr., but no change could be detected between the sheen of the experimental and of the animal controls even after 120 hr. That Euparal has no ill effect on the animal was shown by the fact that one of the controls after being released from its cover-slip was living quite normally in the aquarium more than 3 weeks later. The experiments were repeated several times under the most rigorous conditions, using gas-free 'Nujol' and also using water kept 'boiling' under a filter pump at room temperature for the duration of the experiment. In these instances dead insects were of course used.

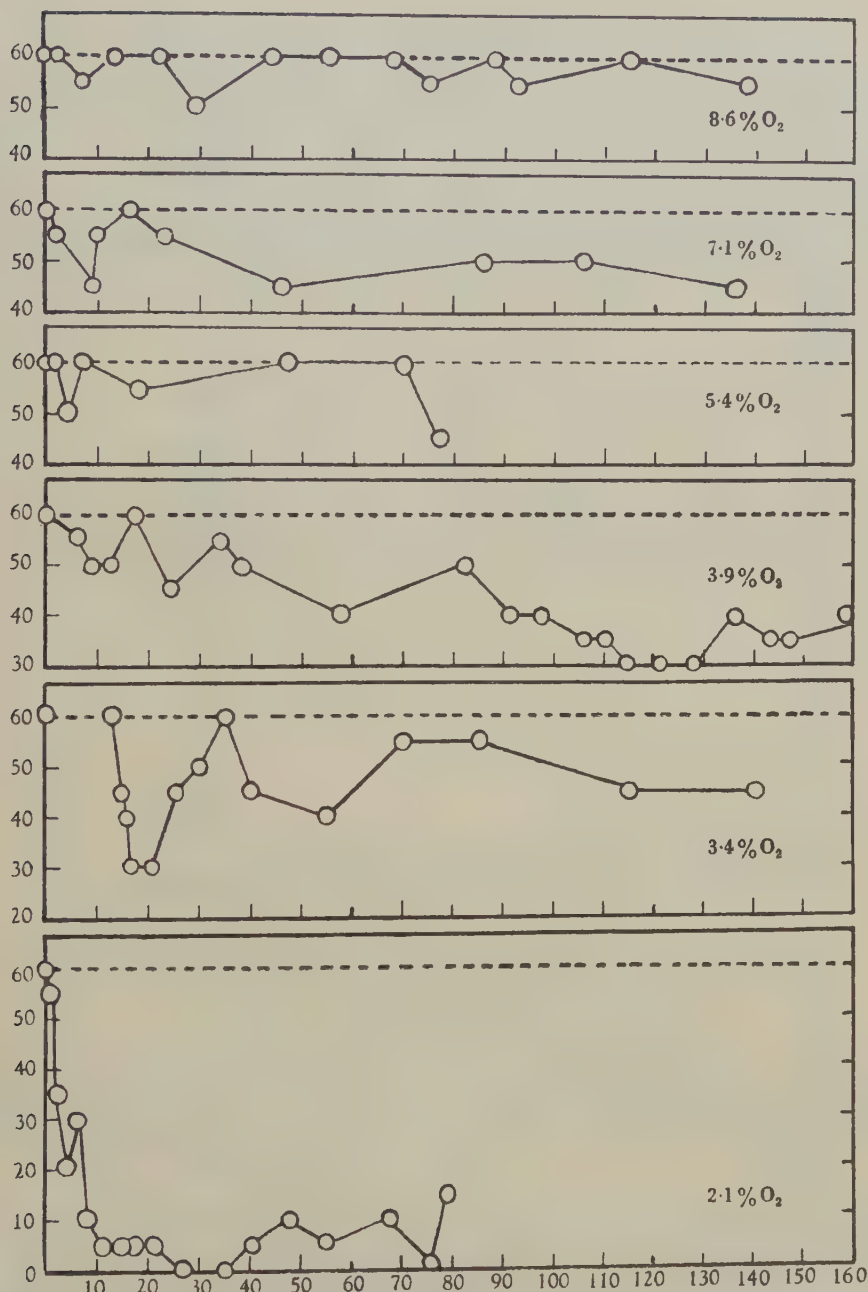
(iii) *Experiments on the respiratory function of plastron*

The next step is obviously to show whether or not the plastron is acting as a gill. For this purpose a simple procedure was evolved of rendering a part or the whole of the plastron ineffective by 'painting out' the plastron under the binocular microscope with a camel-hair brush wetted with cetyl pyridinium chloride or cetyl trimethyl ammonium bromide. This removes the gas layer completely where it is applied and does not show any tendency to spread, providing the insect is quickly rinsed with distilled water. Although these salts are somewhat toxic, a brief application, using a 0.5–1 % solution, followed immediately by rinsing in rapidly running tap water, generally avoided pathological effects. Immediately after operation the

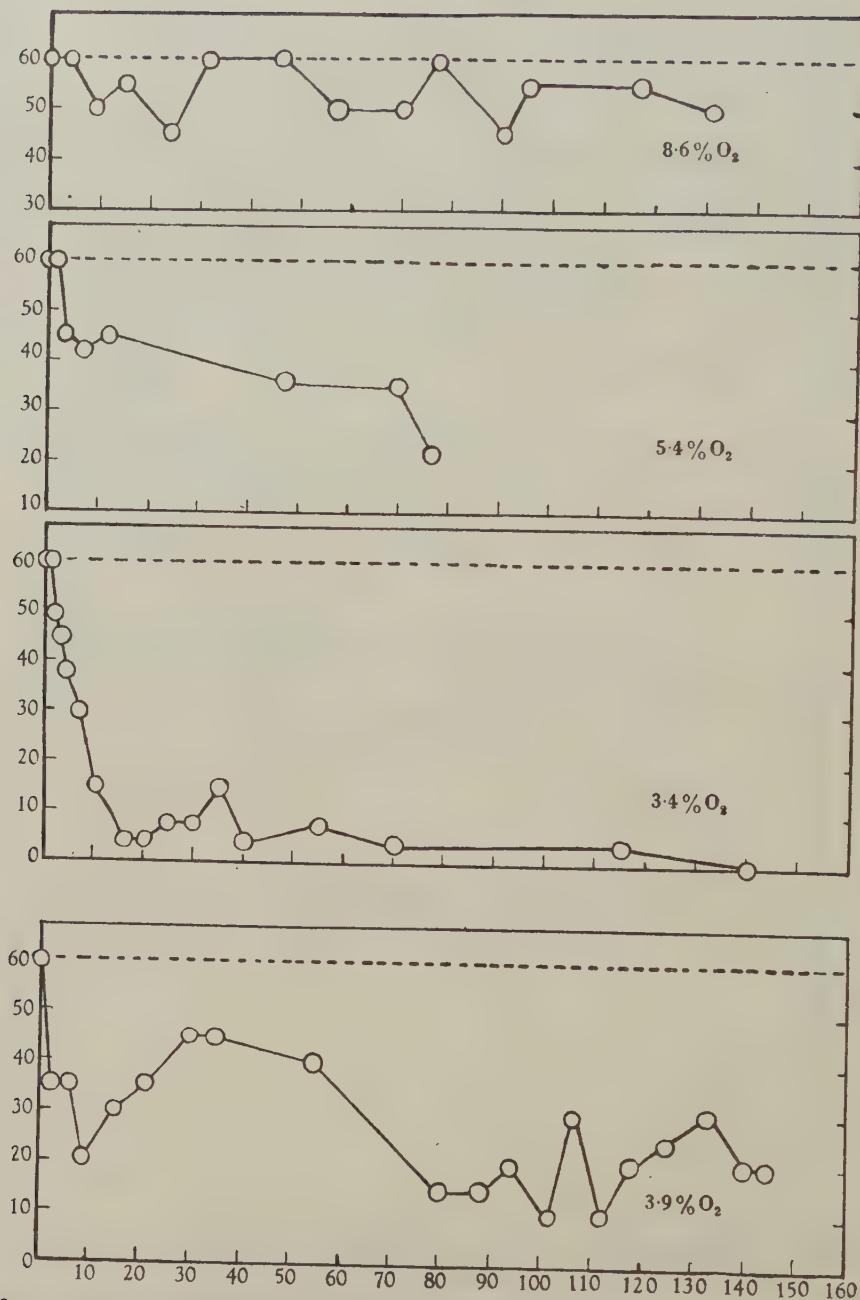
* 'Nujol' is a purified paraffin oil.

bugs were seen to lose some limb co-ordination and to adopt the pose of acute asphyxia, but generally recovery was complete after about 1 hr. The animals were treated on the previous day and allowed to recover overnight, so that any which were damaged and subsequently died were not used in the experiment. Treatment with negative soaps such as sodium dodecyl sulphate and sodium cetyl sulphate was less effective, and concentrations high enough for painting out the air film were found to have definitely toxic effects. Insects thus treated with a positive soap were observed in normal conditions and also subjected to water of known oxygen content less than saturation at atmospheric partial pressure. It was first found that if the whole of the ventral surface of a female were wetted in this way and the animal transferred to 250 c.c. of well-aerated tap water, the insect would become quiescent and show occasional symptoms of asphyxia, but not of acute oxygen want. Controls in which the dorsal surface only was treated showed no significant difference in behaviour from normal animals. The methods were as follows:

A series of four small jars (capacity 200 c.c.), containing 100 c.c. distilled water and a bottom layer of fine sand, were connected with the compressed air system via a mixing chamber to which air and nitrogen were led at a standard rate and constant head through flow-meters. The flow-meters were so adjusted as to give the required gas mixture. Thus, in the experiment where the water contained only 3.5% of oxygen, the air was led through at 2.4 c.c. and the nitrogen at 12.1 c.c./min. This air flow was passed through each of the four containers for some hours before the beginning of the experiment so as to be sure that equilibrium had been established. In the first jar (*A*) were then placed four bugs which had had the whole of the abdominal and thoracic sterna treated, care, however, being taken to avoid penetration of the spiracles by the wetting agent. In the second jar (*B*) were placed the three bugs which had had the abdominal sterna treated, and in the third jar (*C*) three which had had the whole of the tergum only treated. In the fourth jar (*D*) were three control insects. The animals were then observed at intervals of a few minutes and the behaviour classified as follows: N, normal; LA, light asphyxia; AA, acute asphyxia; C, comatose; M, apparently moribund, i.e. completely relaxed and giving no responses. These and many other similar experiments are summarized in graphical form (Text-figs. 8-12) by giving arbitrary scores to the different degrees of asphyxia as follows: N=20, LA=15, AA=10, C=5, M=0. (Since the curves in Text-figs. 8-12 each refer to three individuals, these numbers are multiplied by three.) It will be seen that in most experiments there is initially a period of depression of the treated bugs, which can perhaps be regarded as one of shock. After this is over, however, a clearly marked difference is observable between the experimental and the control animals. Those which have the abdominal and thoracic sterna treated are most seriously affected, those which have the abdominal sterna only treated are only slightly better off, while those which have the whole tergum treated show very little difference from the controls. Text-figs. 10 and 11 show results from a series of experiments where bugs were exposed to mixtures in which the oxygen contents were 5.4 and 3.4% respectively. At the conclusion of the experiments all the bugs showed rapid recovery on removal to well-aerated water. In

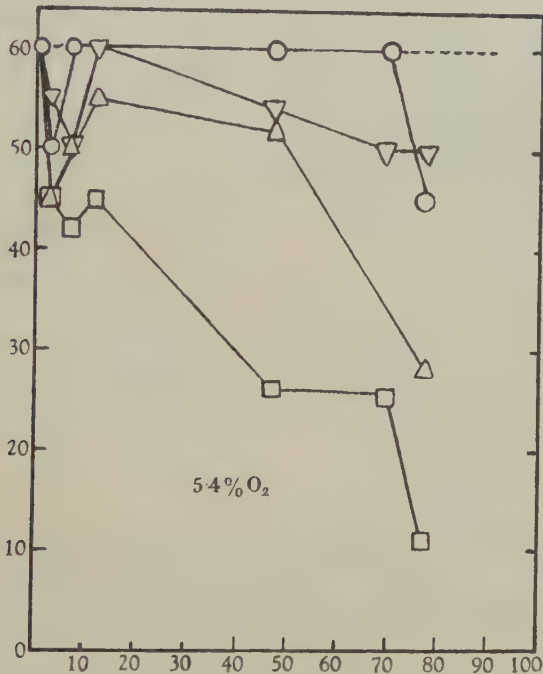


Text-fig. 8. Curves showing tolerance of normal intact adult *Aphelocheirus* to water deficient in oxygen at 18–21° C. Each curve summarizes behaviour of three individuals. Curves commencing at top are in the following descending order of oxygen percentages: 8.6, 7.1, 5.4, 3.9, 3.4 and 2.1%. Horizontal scale shows time in minutes. Vertical scale indicates behaviour as follows: 60 = 'normal'; 45 = 'light asphyxia'; 30 = 'acute asphyxia'; 15 = 'comatose'; 0 = 'apparently moribund'. (For further explanation see text.)



Text-fig. 9. Curves showing tolerance of adult *Aphelocheirus* having whole of ventral surface wetted by painting with cetyl pyridinium chloride to water deficient in oxygen at 18–21° C. Each curve summarizes behaviour of three individuals. Scales as in Text-fig. 8. Curves are in the following order of oxygen tensions: 8.6, 5.4, 3.4% (3.9%, one of the individuals not completely wetted), temperature 18–21° C.

general, those which were less drastically treated and slowest in showing asphyxia were the first to show recovery. Rates of recovery were, however, very erratic, since without constant stimulation it was not possible to determine at what stage the animal was again capable of movement or of assuming a normal attitude. In observing the results of the experiments it was noteworthy that the differences between the animals was often rather a degree of activity than an actual asphyxia attitude. Since it was not possible to express this adequately in the scores the results are in fact more striking than the curves based primarily on the attitude would indicate. From these experiments we can conclude that simple body-surface respiration, without the intervention of any plastron, will suffice, under

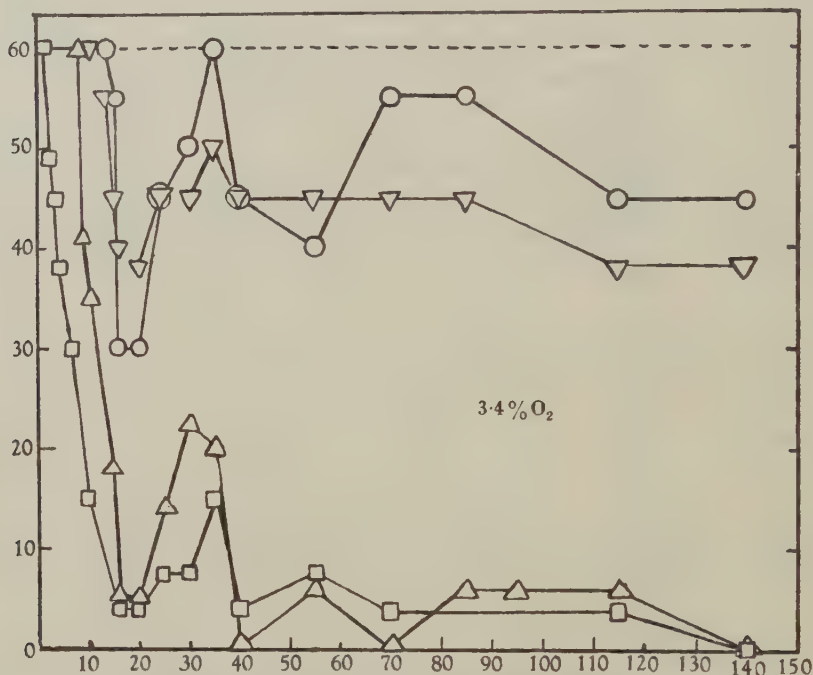


Text-fig. 10. Curves showing effect on tolerance of adult *Aphelocheirus* to water at 5.4% oxygen, of having had different parts of the body surface wetted with cetyl pyridinium chloride. ○ = control. ▽ = terga only wetted. △ = abdominal sterna only wetted. □ = thoracic and abdominal sterna wetted. Scales as Text-fig. 8. Each curve represents three individuals. Temperature 18–21° C.

good conditions, when the animal is quiescent, for the prevention of all except the mildest degree of asphyxia. Such respiration will include, of course, a small amount of invasion at the spiracles themselves, since these were not actually blocked. But an intact plastron becomes vitally important for the maintenance of normal activity and for survival when the oxygen tension is appreciably reduced below saturation at atmospheric partial pressure. These results were fully confirmed by respirometer experiments to be described in the next paper in the series.

(b) *Comparison with nymph*

Text-fig. 12 shows for comparison the tolerance to oxygen deficiency of well-grown fifth instar *Aphelocheirus* nymphs studied in the same way. As will be shown in a subsequent account of the tracheal system of the nymph, the respiration of the early stages is on a different basis from that of the adult. The former has a completely closed tracheal system and lacks the plastron, and, being smaller and very flat, exposes a large surface to the water. In addition, the lateral margins of the abdominal segments are greatly produced and richly supplied with fine tracheae, thus presumably



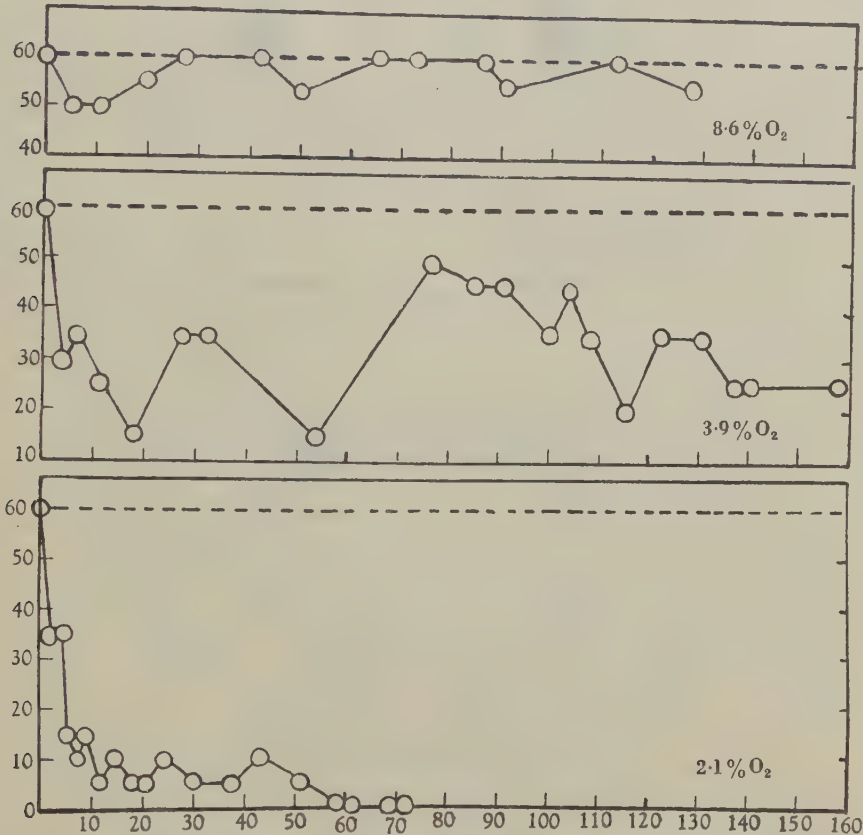
Text-fig. 11. Curves showing tolerance of partially wetted adult *Aphelocheirus* to water with oxygen tension at 3.4%. For explanation see Text-fig. 10.

acting as tracheal gills (see Text-figs. 13-17). The cuticle, too, is distinctly more transparent than in the adult. The difference in weight between a well-grown fifth instar nymph and an adult is, of course, considerable; thus an average weight of a living adult, air dried, is 0.04 g. (average of five individuals), whereas a well-grown fifth instar nymph, not yet ready for moult, weighs about half this amount. The nymphs are on the whole less active than the adults, and it appears that when the insect is of the size at which it is due to assume the adult condition, a critical period is reached, at which simple diffusion across the cuticle is no longer adequate for respiration. This question will be considered in greater detail in a later paper.

5. DETAILED INVESTIGATION OF THE PLASTRON HAIRS AND THEIR RELATION TO THE CUTICLE STRUCTURE AS A WHOLE

(a) *The shape and size of the plastron hairs*

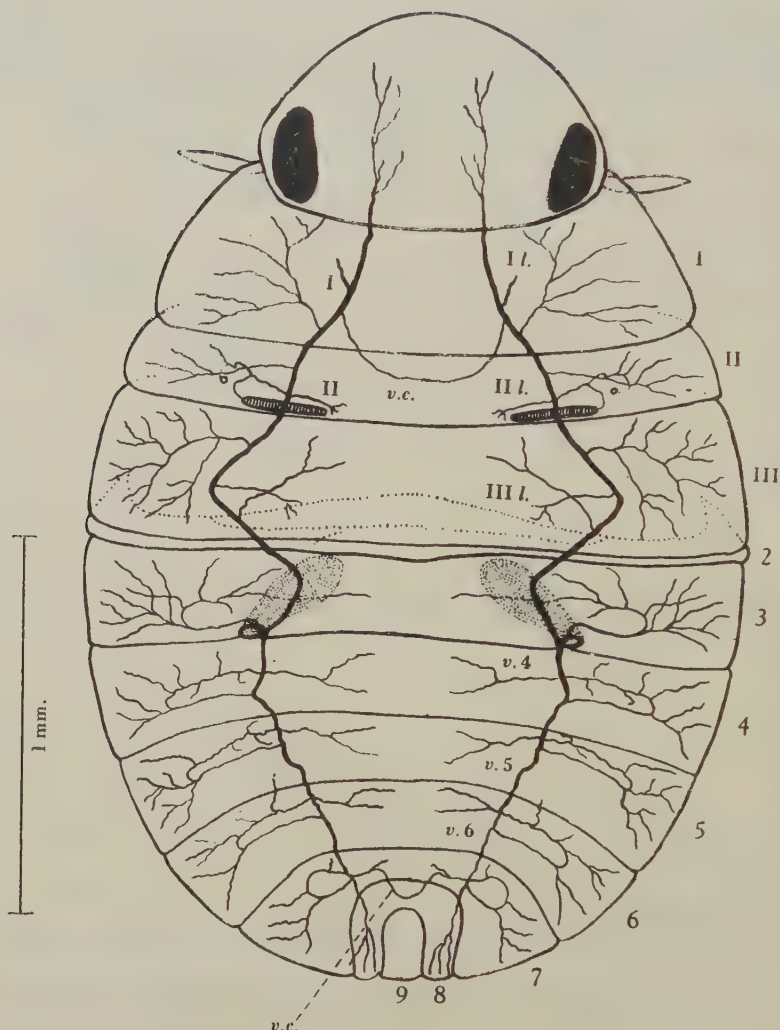
In order fully to understand the way in which the plastron hairs hold the gas, and in order to appreciate the limitations of plastron respiration, it is necessary to know the exact shape, number and dimensions of the plastron hairs. To determine this has proved to be a much more difficult matter than was at first expected. The hairs are



Text-fig. 12. Curves showing tolerance of normal intact fifth instar nymphs of *Aphelocheirus* to water deficient in oxygen. For explanation see Text-fig. 8.

not stainable but show in section as pale yellow structures. They are so minute as not to be clearly visible except under an oil-immersion lens with a high-power ocular. They show rather poorly in balsam, and somewhat better in Euparal and better still in biniodide of mercury and potassium, a medium which has been found valuable for examination of minute detail in protistan skeletons. The usual method of examination was to make sections with the freezing microtome and to examine them under an oil-immersion lens with an Ediswan 150 c.p. 'Pointolite' and a Wratten

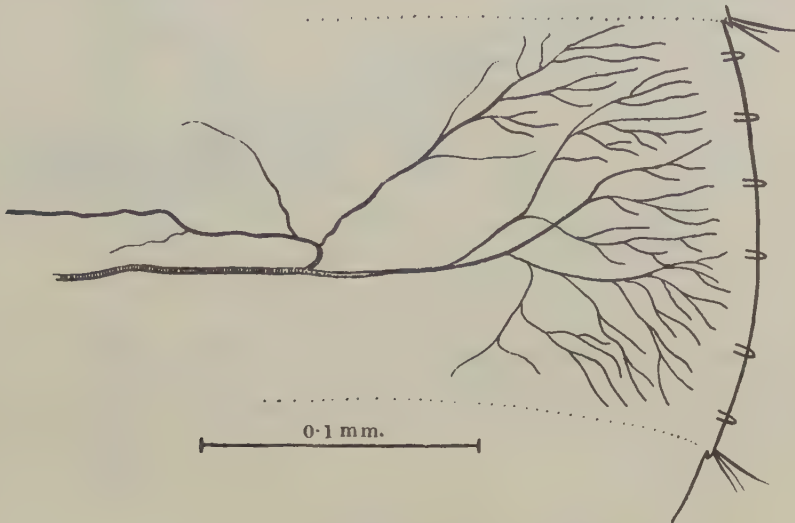
H Filter. Under these conditions a very close-set pile of hairs is visible (Text-fig. 18), arranged with the utmost regularity and neatness. (In many parts of the section the air will, if alcohol or any other wetting agent has not been used, still be present in a large part of the hair pile, and there, of course, the hairs cannot be seen.) Under the



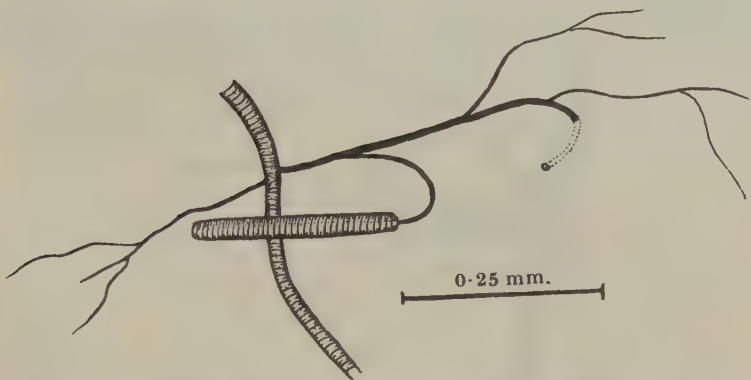
Text-fig. 13. First instar nymph of *Aphelocheirus* to show tracheal system. Only the main twigs of the dorso-lateral branches are shown. *II l.*, *III l.* and *III l.* = branches serving legs 1, 2 and 3. *v. 4-v. 6* = ventral branches of abdominal segments 4-6. *v. c.* = ventral commissure. (Body surface sensillae omitted.)

best conditions of mounting and lighting and with a high-power ocular the hairs can be counted and drawn, but even so it is impossible to arrive at a convincing measurement of their thickness, which appears to be rather less than the wave-length of ordinary light and therefore at the limit of resolution. It is, however, clear (see Text-fig. 19) that where unharmed they stand perfectly erect, making an angle of

90° with the cuticular surface, and that they are neatly bent over at the tips, all in the same direction. A series of careful counts of sections gives an estimate of their numbers at approximately $2\frac{1}{2}$ million per sq.mm. In certain protected places, e.g. the spiracular grooves of the thorax and the channels of the spiracular rosettes

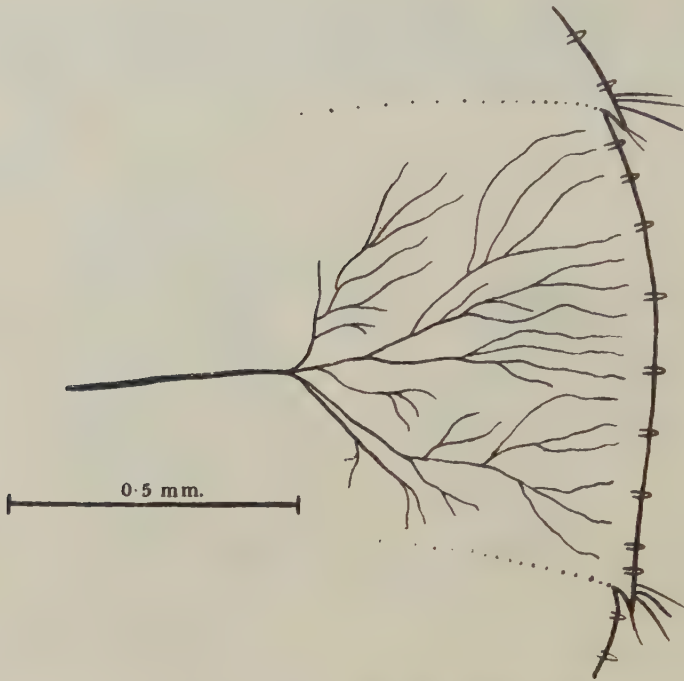


Text-fig. 14. Lateral margin of fourth abdominal segment of first instar nymph of *Aphelocheirus*, showing dorsal branch of trachea.

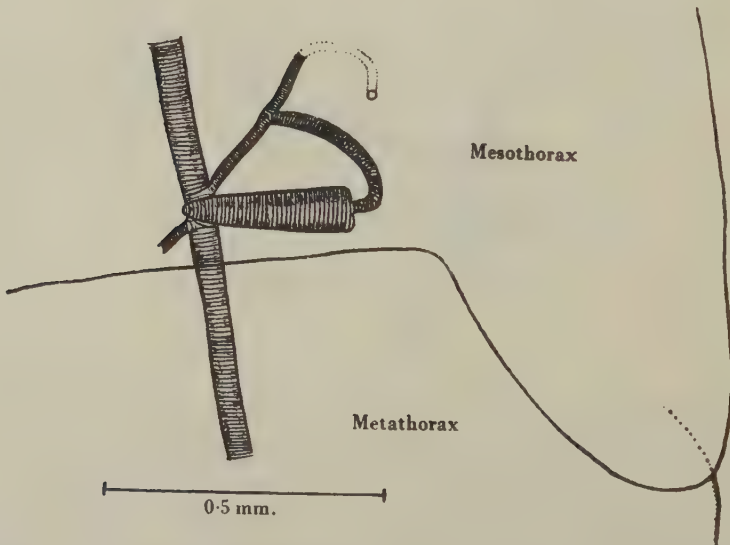


Text-fig. 15. Air sac and associated trachea of first instar nymph of *Aphelocheirus*.

(see Text-figs. 4, 20), the plastron hairs are longer than they are on the more exposed surfaces of the thoracic and abdominal sterna, but the essential structure appears the same. In order to get further information as to the thickness and dimension of the tips of the hairs, studies were made with the electron microscope. With this instrument two methods were employed. First, from among a series of sections freshly cut with the freezing microtome, some were picked out which tapered

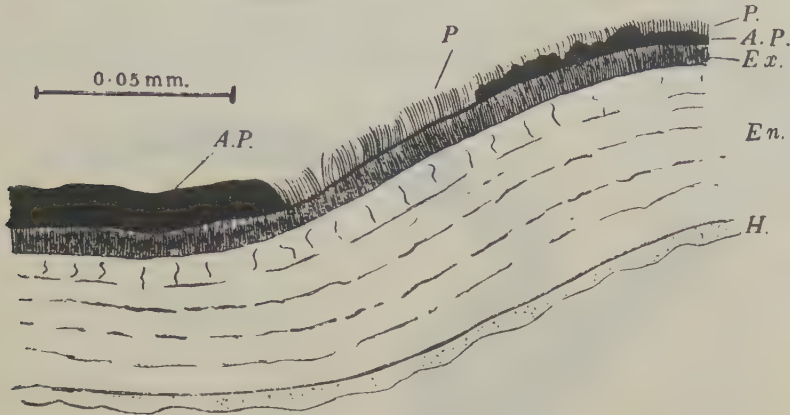


Text-fig. 16. Lateral margin of fifth abdominal segment of fifth instar nymph of *Aphelocheirus* showing dorsal branch of trachea.

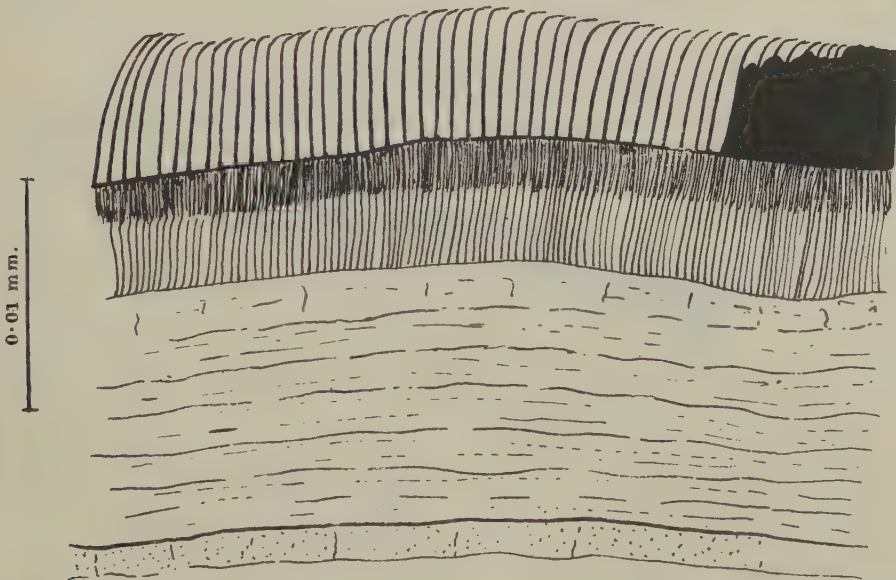


Text-fig. 17. Air sac and associated trachea of fifth instar nymph of *Aphelocheirus*.

toward the edge and thus tended to have very thin margins. These were teased and pulled with fine needles under the dissecting microscope so as to increase the chance of separating out single hairs and the section was then placed on the grid. Here and



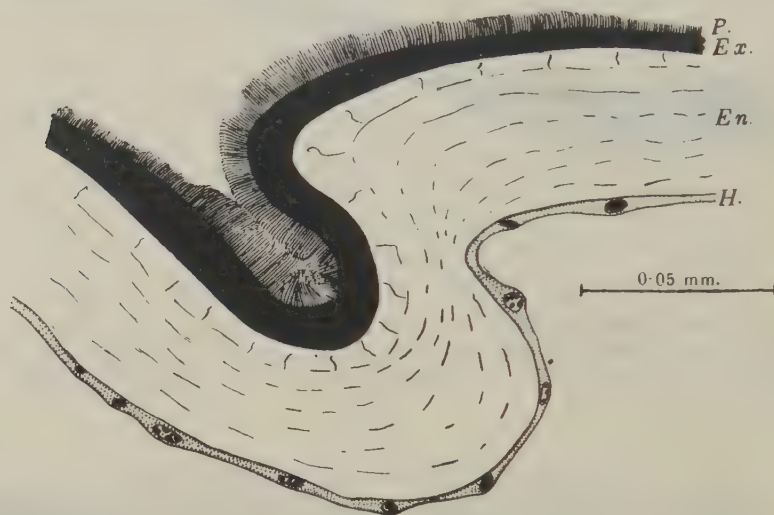
Text-fig. 18. Fresh unstained section of abdominal sternum of adult *Aphelocheirus* made with freezing microtome, mounted in 'Biniodide' and observed by transmitted light. *Ex.* = exocuticle. *En.* = endocuticle. *H.* = hypodermis. *P.* = plastron hair pile. *A.P.* = region of plastron where air is still present.



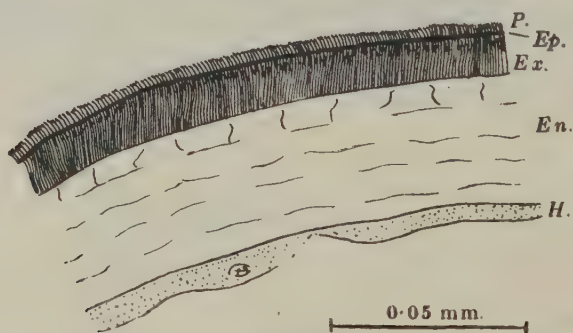
Text-fig. 19. Similar section to Text-fig. 18, observed under highest obtainable power of the light microscope.

there the tip of a single hair was visible under the electron microscope as a silhouette (Pl. 7, figs. *g*, *h*). Secondly, the surface of the abdominal sterna was studied by means of the gelatin-formvar replica technique. In using this method the sterna were covered by a thin layer of 10 % solution of gelatin and left to dry hard. The

gelatin was then cracked off and coated very thinly with a solution of formvar in dioxan, which dried rapidly. The specimen was then placed in slightly alkaline water to dissolve the gelatin away, and the formvar film floated on to the grid for examination. A true replica was not obtained, since the hairs are extremely long and thin for such a method. On cracking off the gelatin, however, the embedded hair tips were plucked out, and a considerable number attached themselves to the formvar



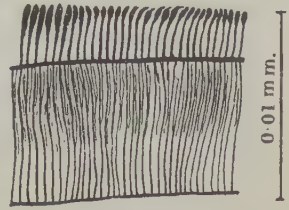
Text-fig. 20. Section through one of the grooves which connect with the thoracic spiracles on the ventral surface of thorax to show variation in length of plastron hairs. Lettering as Text-fig. 18.



Text-fig. 21. Section through abdominal tergum of adult *Aphelocheirus*. Lettering as Text-fig. 18. *Ep.* = epicuticle.

film. Pl. 7, figs. *g* and *h*, show the features which are revealed by these methods. In spite of the many difficulties encountered they show fairly clearly the tips of a number of hairs and the diameter works out at $0.21-0.25 \mu$. Because of the difficulty of obtaining sections thin enough to be transparent to the electron beam, and because of the difficulty of orientating material in the field of the electron microscope, the hair structure was also investigated by means of an ultra-violet light microscope.

This has given very satisfactory results and has confirmed the previous estimate of dimensions and number of the hairs. Pls. 6 and 7, figs. *a-f*, give a selection of photographs of the plastron hairs taken by this method. From this it is estimated that the diameter of the hairs on the ventral surface is 0.18μ , the distance apart (i.e. the distance between cores) 0.6μ , and the space between adjacent hairs approximately 0.4μ . Counts based on the ultra-violet light photographs give a result similar to that obtained with a visible-light microscope, namely, $2\frac{1}{2}$ million per sq.mm., although some estimates based on a surface view under oil immersion suggest that in some regions the density is less than this, being round about 2 million. The total area of ventral surface of the adult, including the head, is approximately 36 sq.mm. The hairs on the dorsal surface are shown in Text-fig. 21. Here they are often not so regular and do not appear to bend over at the tips in the same neat way as those found on the sterna, though in some regions they appear slightly clubbed at the tips (see Text-fig. 22). The whole cuticle is much more deeply pigmented, with the result that the terga have not the same sheen as the sterna, but it is doubtful if there is any fundamental difference between the two.



Text-fig. 22. Plastron hairs, epicuticle and exocuticle only as seen in a frozen section of abdominal tergum of adult *Aphelocheirus*.

(b) Chemical and physical characteristics of the plastron hair system

It will be seen from Text-figs. 18–22 that in the adult there is a thick endocuticle lying above the hypodermis, this endocuticle being composed of five or six distinct layers, the outermost of which shows rather distantly spaced cross-striations. The endocuticle contains practically no pigment. The exocuticle is heavily pigmented. In some sections little structure can be seen in it, but in others innumerable fine transverse lines can be observed, which appear to be pore canals. These are shown in Text-fig. 19. Only rarely is it possible to see where these pore canals enter the endocuticle. The epicuticle is very difficult to distinguish from the exocuticle, but it appears in most sections as a faint line bounding the surface from which the plastron hairs arise. It is of course impossible, by ordinary microscopical methods, to observe any structure in the plastron hairs themselves, because of their minute size. Campbell's concentrated $\text{KOH-H}_2\text{SO}_4\text{-KI}$ method produces a good chitin reaction both in the exo- and endocuticle but none at all in the epicuticle or plastron hairs. The coloration in the exo- and endocuticle is quite general and does not appear to be confined to pore canals. In no case was staining of the pore canals obtained, yet in a few cases in sections of adults, pore canals were rendered visible in the endocuticle after treatment with 1% H_2SO_4 , following the potash treatment applied in Campbell's method for chitin. The best of these sections were photographed and both exo- and endocuticle pore canals can be made to show well, although a different exposure is necessary for the two. It appears from these photographs that pore canals are more numerous in the exocuticle than in the endocuticle. It is difficult

to be sure whether or not this means that the canals branch at the junction of the two layers. In many sections pore canals could be seen in some layers of endocuticle but not in others. Often they were conspicuous in alternate layers, but it is not at present clear what significance this may have. It is interesting to note that Dennell (1946) finds pore canals present only in the older outer layers of the endocuticle of the larva of the fly, *Sarcophaga falcitata*, no trace being observable in the inner layers. The interpretation of sections after this drastic potash treatment is often difficult. The exocuticle tends to break away from the endocuticle, and different layers of endocuticle tend to split and form a tangled mass of almost structureless fibres. Yet in spite of this the plastron hairs will often survive the treatment, clumped and appearing partly fused but still visible. Little is known then of the physical properties or of the chemical nature of the epicuticle, of which the hairs appear to be composed. From its great resistance to chemical action it is likely to be a highly cross-linked substance, most probably protein, with perhaps some chitin.

As will be seen from some of the photographs taken by ultra-violet light there is often an appearance which suggests that the plastron hairs are essentially continuations of structures within the exocuticle. Pls. 6 and 7, figs. *c*, *d*, *e*, in particular, show what appear to be the 'roots' of the plastron hairs passing through several horizontal layers in the outer part of the exocuticle—layers which are not visible at all by any other method we have tried. The fact that these 'roots' absorb ultra-violet radiation more strongly than their surroundings suggests a protein framework. It is interesting in this connexion that Hurst (1945) on the basis of permeability studies has postulated a mosaic structure of the insect cuticle very much of this form. It is also likely on general grounds that there is a molecular orientation along the axis of the hair. The structure of the nymphal cuticle and its relation to the question of development of the plastron in *Aphelocheirus* will be discussed in the second paper of this series.

It was noted quite early in experiments with *Aphelocheirus* that the tips of the plastron hairs of newly emerged adults, that is, the surface of the plastron itself, seemed more hydrophobic than with older animals. It was also observed that if an adult bug was taken from water and carefully dried with filter-paper and then kept dry for half an hour, the hydrofuge properties of the plastron surface seemed enhanced.

As mentioned above cetyl pyridinium chloride proved to be a satisfactory wetting agent when it was desired to remove the plastron or any part of the plastron as a respiratory structure without harming the animal. It was instructive for some purposes to watch the spread of oils such as clove oil and medicinal paraffin (Nujol) over the plastron surface, but these were not satisfactory for experiments in that their spread could not be conveniently arrested. A number of experiments were carried out to determine the wetting power of solutions of pure isobutyl alcohol in water at 18° C. It was found that solutions up to 8% strength had no effect whatever. Above this strength, changes in appearance of the plastron might commence slowly but had not proceeded far in 5 min. Solutions between 9 and 11% had a more marked effect, in some specimens the sheen disappearing fairly quickly at 10%, in

others very slowly, but even in the most resistant specimens the change proceeded rapidly at 12% and was easily noticeable after 30 sec. exposure. Full wetting with butyl alcohol caused a dull black colour to replace the sheen, and no restoration could be achieved even in tap water (supersaturated with air). When washed, dried and reimmersed in water the sheen was fully recovered and no change in properties could be noticed, resistance to wetting being in no way diminished. Hence it is not likely that butyl alcohol exercises any irreversible change on the structure of the hair pile beyond normal wetting. The well-known difference between advancing and receding contact angles is sufficient to explain the irreversibility of the process as long as the system is kept immersed.

It has already been shown that the plastron hairs are not wetted by immersion in gas-free distilled water. The next question is, what hydrostatic pressure is necessary forcibly to wet the plastron with gas-free water? In order to determine this, a piece of abdomen including sterna of the anterior segments was subjected to increased pressure in distilled water in an apparatus consisting of a stout pressure jar and a manometer. In order to observe accurately the change in sheen, it was necessary to have the specimen appropriately orientated in good diffuse daylight (various sources of artificial light were tried but all were inferior to daylight) and to have a control specimen in a similar chamber beside it for comparison, orientated in the same way. Experiments were repeated many times, and it was found that with a fairly young bug with a good sheen the glint began to fade at about 3 atm. excess pressure, although the margins of the segments were still at full sheen. The sheen vanished entirely and immediately at a pressure of between 4 and 5 atm., but it was noticeable that the specimen did not acquire the dull black appearance which is produced by wetting with butyl-alcohol solutions. If the pressure was released and the specimen now transferred to aerated distilled water or to tap water, some recovery of sheen was shown in about 10 min. Complete recovery did not appear to take place as long as the specimen was immersed. If, however, it was dried and then returned to water, its appearance became completely normal again. In another experiment the specimens were kept at a pressure of 2 atm. for a longer period. In this case no marked reduction in sheen took place, though there was a barely perceptible change after a period of 5 hr.

When the hair pile is exposed to increased pressure in a fluid of lower contact angle and surface tension than pure water, wetting should take place at a lower value than those just described. The experimental points on Text-fig. 26 were obtained by exposing the bug to excess pressure in distilled water saturated with air and containing different concentrations of butyl alcohol, whose contact angles against wax had previously been determined (see Text-fig. 25).

The difference in appearance and in tendency to recover sheen as between plastron wetted by fluids of low-surface tension on the one hand and by pure water under excess pressure on the other, indicates that we have here two entirely different phenomena. In the first case we have a true wetting brought about by the entry of the fluid in between the hairs thus displacing the gas. In the second case the evidence strongly suggests that the excess pressure causes a collapse of the hair pile itself.

When external pressure is applied to the hair pile it is not only necessary that the surface forces should prevent entry of water into the plastron, but it is also necessary that the hair pile itself should be capable of withstanding the pressure difference. If this were not so the hairs would collapse and the air space be eliminated.

(c) *Mechanical theory of the resistance of the plastron to wetting*

(i) *Theory of wetting by surface forces*

We shall now examine the conditions under which a system of hairs would be theoretically capable of maintaining an air film against excess pressure outside, and calculate the order of magnitude of the pressure difference involved. The reader is referred to N. K. Adams *Physics and Chemistry of Surfaces*, 3rd ed. 1941, pp. 177-201 (Clarendon Press), for the general principles underlying this section.

The simplest system which can be envisaged is one of vertically placed cylindrical equidistant hairs making a contact angle θ to the water surface (Text-fig. 23a). If the radius of a hair is r , the number of hairs n per sq.cm., and mean distance apart l , so that $n = \epsilon l^{-2}$ (where ϵ is a factor dependent on the type of packing; in the case, here assumed, of rectangular packing, $\epsilon = 1$) then the virtual work for a displacement of the water level by a distance dx will be

$$2\pi r n W dx = \frac{2\pi r W dx}{l^2},$$

where W is the adhesion tension $\gamma \cos \theta$, γ being the surface tension of water.

The work done against external pressure difference Δp will be

$$(1 - \pi r^2 n) \Delta p dx = \left(1 - \frac{\pi r^2}{l^2}\right) \Delta p dx.$$

The hairs will just be wetted if the value of Δp is critical so that

$$\begin{aligned} \frac{2\pi r^2}{l^2} W dx &= \left(1 - \frac{\pi r^2}{l^2}\right) \Delta p dx \\ \text{or } \Delta p &= \frac{2\pi r \gamma \cos \theta}{l^2 - \pi r^2}. \end{aligned} \quad (1)$$

If the hairs are inclined at an angle α to the horizontal, the above expression becomes modified to

$$\Delta p = \frac{2\pi r \gamma \cos \theta \operatorname{cosec} \alpha}{l^2 - \pi r^2 \operatorname{cosec} \alpha}. \quad (2)$$

The second system which will be considered is that in which the hairs are cylindrical, and lie in a parallel series as shown in Text-fig. 23c, d.

It can be shown from the geometry of the system that, since the amount of surface of hair covered by the water varies non-linearly with the lowering of the water surface, the excess pressure in equilibrium with the surface forces gradually increases as the water surface is lowered and the water 'bubble' is forced into the space between the hairs. This excess pressure passes through a maximum and decreases again, just as the inflation through a tube of a spherical bubble passes through a pressure maximum.

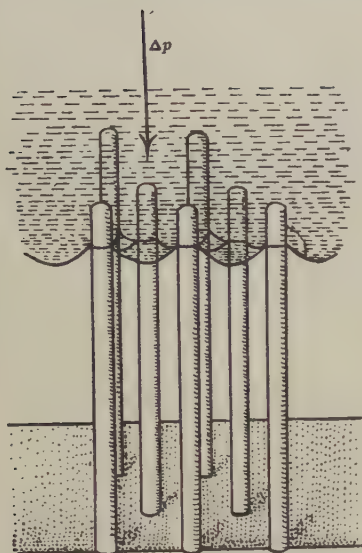


Fig. 23a

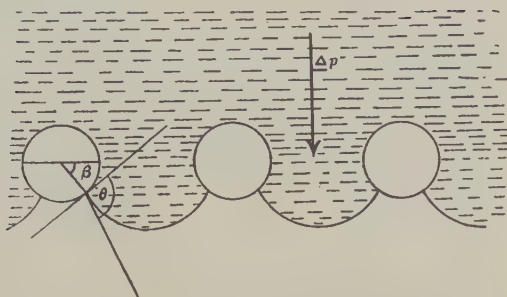


Fig. 23b

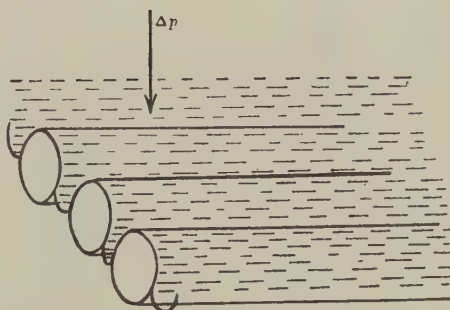


Fig. 23c

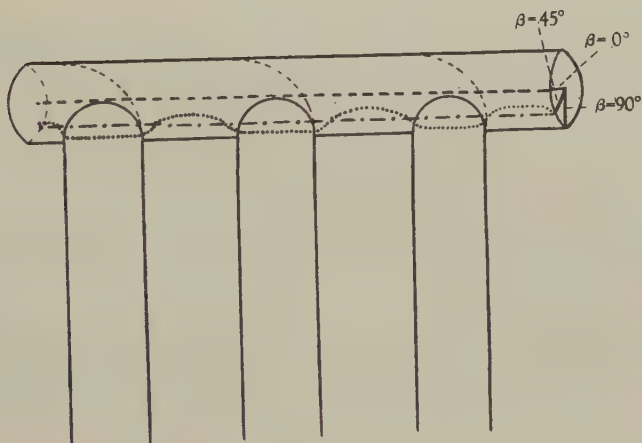


Fig. 23d

Text-fig. 23. Diagrams to illustrate the wetting of: (a) a system of cylindrical hairs which are straight, erect and equidistant. (b), (c) a system of cylindrical hairs which are straight, horizontal and in parallel series. (d) a combined system of vertical and horizontal hairs. (For further explanation see text.)

The excess pressure Δp for the system, derived in a manner analogous to that of equation (1), is

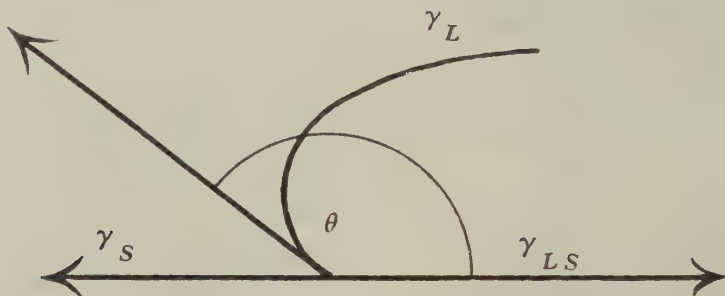
$$\Delta p = \gamma \frac{\cos(\beta + \theta)}{\frac{1}{2}l - r \cos \beta}, \quad (3)$$

β being the angle between the horizon and point of wetting of the hairs, and assumed positive when more than half the hair is wet (see Text-fig. 23*b*), and θ the contact angle between hair surface and water surface.

The maximum pressure sustained depends on the ratio of radius of hair r and separating distance l , for

$$\max. \Delta p \frac{\sin \theta + \beta}{\sin \theta} = \frac{2r}{l}. \quad (4)$$

The value of θ and γ must next be considered in applying these equations to the hair structure of an insect.



Text-fig. 24. Diagram to show the angle of contact, θ , made by a sessile drop of fluid on a solid surface, where γ_S is the solid-air tension, γ_{LS} the liquid-solid tension and γ_L the liquid-air tension. (For further explanation see text.)

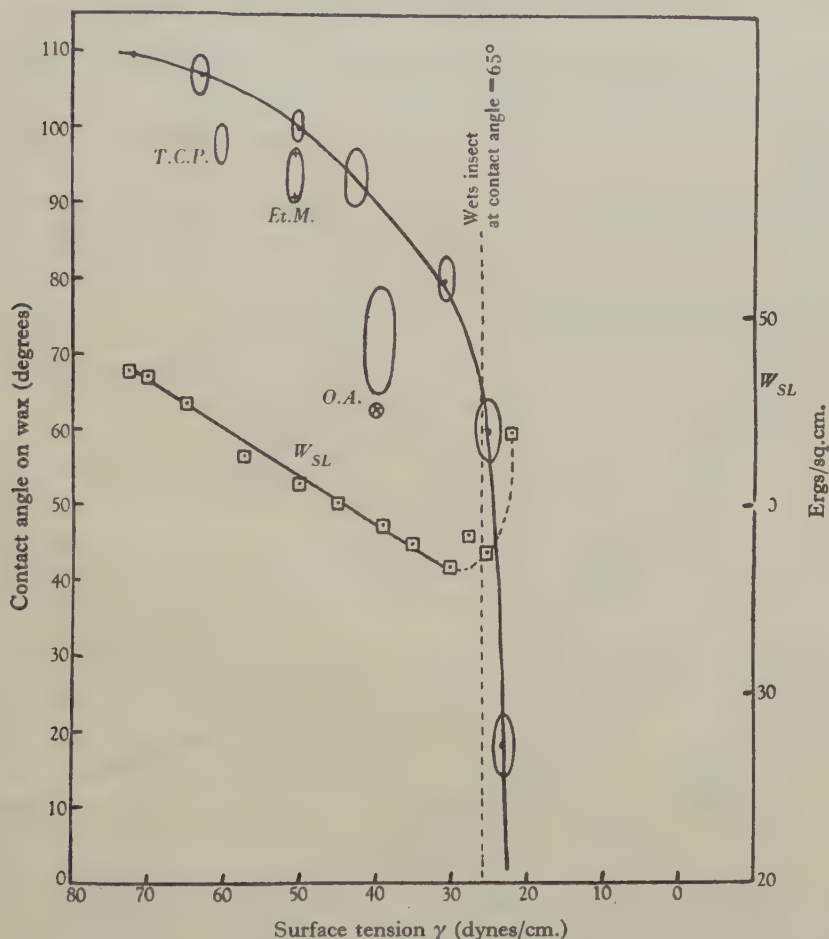
Since the hairs have a hydrophobic surface, either on account of their innate structure or due to a waxy layer, the outer surface is likely to be composed mainly of hydrocarbon groups. The contact angle is dependent only on the molecular configuration of the outer layers, and if these are hydrocarbon the value of θ lies in the region of $105-110^\circ$. If the surface is rough or cavitated there will be an apparent increase in θ (Wenzel, 1936; Cassie & Baxter, 1944), but this factor will be ignored for the present and a value of 110° taken, the surface being assumed to be similar to that of solid hydrocarbon. Electron microscope pictures of the hair indicate no roughness of their surface.

This value of θ refers to the contact angle against a clean water surface. If the surface tension of the water is altered, θ will change on account of the alteration in the forces of the Neumann triangle, but the change in θ is not in the expected sense. In Text-fig. 24 it can be seen that

$$\gamma_S - \gamma_{LS} - \gamma_L \cos \theta = 0,$$

where γ_S is the solid-air tension, where γ_{LS} is the liquid-solid tension, where γ_L is the liquid-air tension. A reduction in γ_L caused by an impurity would be expected to cause θ to increase if $\theta > 90^\circ$. In practice, however, θ always decreases, probably because adsorption occurs simultaneously at the liquid-solid boundary,

decreasing γ_{LS} . It will be observed from the graph (Text-fig. 25) that the adhesional work ($\gamma_L + \gamma_S - \gamma_{SL}$) varies slightly and linearly with the surface tension over a greater part of the curve, suggesting that adsorption is similar at the air-water and hydro-carbon-water interface. The relation between γ_L and θ is not necessarily similar for all impurities, but depends on the equilibrium values of γ_S and γ_{LS} when an impurity is present. The θ - γ relation for three insoluble oils is also given.



Text-fig. 25. Surface tension (γ dynes/cm.) contact angle (degrees) curve, isobutyl alcohol solution on paraffin wax (m.p. $56^\circ\text{C}.$). *O.A.* = oleic acid. *T.C.P.* = tricresylphosphate. *Et.M.* = ethyl myristate. Adhesional work $W_{SL} = \gamma_L + \gamma_S - \gamma_{SL}$ is also plotted.

The values of θ were obtained by comparing the shapes of small sessile drops containing known concentrations of butyl alcohol on a paraffin block. The contact angle θ is given by

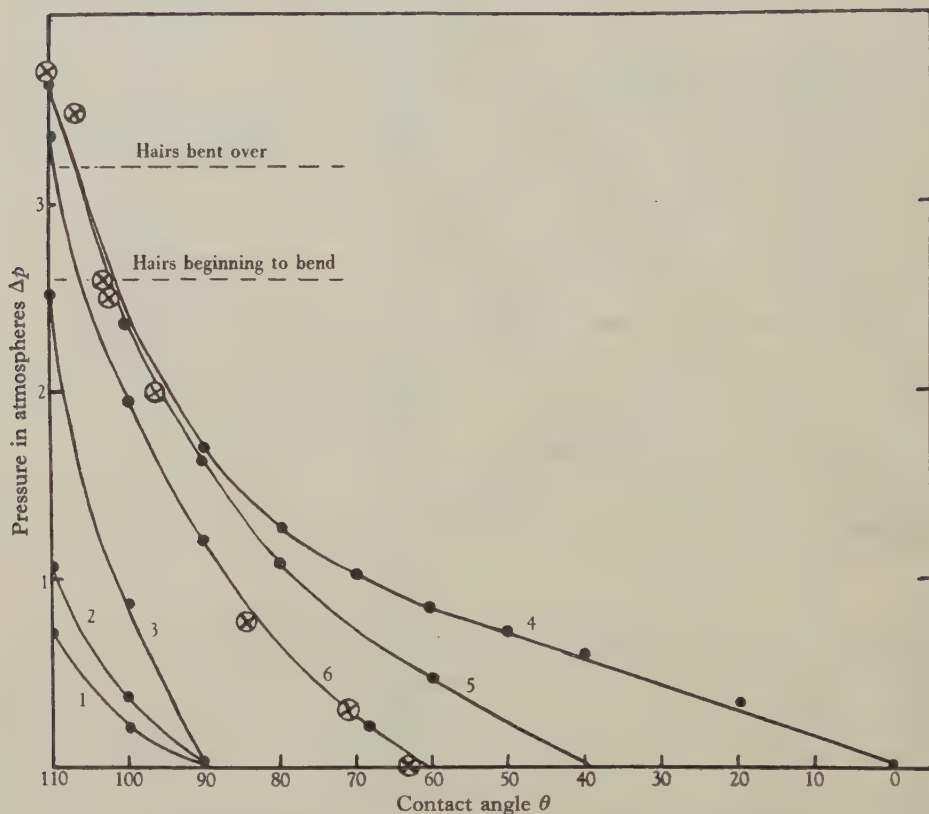
$$\frac{\Delta^3}{v} = 24 \sin^3 \theta / \pi (2 - 3 \cos \theta + \cos^3 \theta)$$

(Bikerman, 1940), where Δ = length of base of droplet and v = volume of droplet measured by an Aglar microsyringe.

The surface tension was measured by the ring method applying Harkins's corrections (Harkins & Jordan, 1930). In order to apply the equations to conditions under which θ might vary on account of impurities we made the following assumptions:

(a) That the surface of the hair resembled that of a freshly cut block of pure paraffin wax of m.p. 56°C .

(b) That butyl alcohol may be taken as a typical surface active impurity.



Text-fig. 26. Curves to show excess pressure (Δp in atmospheres) sustained by hair pile of dimensions found in *Aphelocheirus* as a function of the contact angle. 1, vertical hairs (equation (1)). 2, hairs inclined at an angle of 45° to horizon (equation (2)). 3, hairs inclined at an angle of 30° to horizon (equation (2)). 4, horizontal hairs; limiting value of angle 90° (equations (3) and (4)). 5, horizontal hairs; limiting value of angle 50° (equations (3) and (4)). 6, horizontal hairs; limiting value of angle 30° (equations (3) and (4)). Experimental data (see text).

Applying these values of θ and γ to equations (1), (2) and (3) we obtain the curves shown in Text-fig. 26, assuming the dimensions $r = 1 \times 10^{-5}$, $l = 5 \times 10^{-5}$ cm. for the hair pile of the bug *Aphelocheirus*.

It is apparent from the graph that the least efficient type of hair pile is one in which the hairs are directed vertically to the surface (curve 1). Curves 2 and 3 ($\alpha = 45^{\circ}$ and 30°) indicate the improved efficiency against wetting which is obtained by

inclining the hairs at an angle. The hairs are still wetted when θ is $< 90^\circ$, however, and there is a limit to the angle α , since eventually the hairs would touch one another. For instance, if the r/l ratio is 1:5, the hairs would touch if inclined at an angle of less than 25° .

Curve 4 indicates the pressure necessary to wet a system of purely horizontal hairs; it will be noted that a much greater pressure is required to wet the hairs, and that a positive pressure is necessary for all contact angles above zero. This is clearly a far more efficient system.

In practice, of course, it is not possible to have a system of horizontal hairs without support. Vertical hairs in which the tips are sharply inclined at 90° would offer the best approach to such a condition and this is what is in fact found in *Aphelocheirus*.

The effect of the vertical supports to the horizontally placed hair tips will naturally cause the behaviour to show some deviation from that predicted from equations (3) and (4) and shown in Text-fig. 26, curve 4. We should anticipate that on increasing the angle θ the line of contact of the water surface will not remain straight when $\beta > 0$, but will assume a contour roughly as shown in Text-fig. 23*d*. It is impossible to apply any mathematical analysis to this system, but it is clear that for contact angles θ greater than 90° , the vertical portions will draw the water surface downwards and so cause instability and wetting at a value of β which is less than 90° , possibly much less. When this unstable point is reached the menisci will follow the vertical portions and cause immediate wetting and waterlogging of the plastron. If θ is greater than 90° , however, the vertical regions will also resist wetting and their effect will not be to cause instability. Text-fig. 26, curves 5 and 6, show the effect if the system is assumed to become unstable at a value of β of less than 90° ; curve 5 is for $\beta < 50^\circ$, and curve 6 for $\beta < 30^\circ$. The experimental points shown on the same graph were obtained as described on p. 255 above. They indicate clearly that the hair pile functions as a horizontal system, but instability rapidly sets in as predicted above, when the contact angle is less than 90° . Above 90° contact angle the points agree very well with those calculated from equation (3). Although the precise agreement found is probably somewhat fortuitous, since Δp is very sensitive to the density of packing of the hairs (which will be altered if there is any considerable overlap), there is no doubt that agreement exists both in respect of the order of magnitude of Δp and the anticipated relation between Δp and θ .

When θ falls below 90° the agreement breaks down and wetting occurs at zero pressure when θ is just above 60° . This is doubtless due to the presence of the vertical supports. It will be seen that the experimental points in this region agree well with curve 6, calculated on the assumption that instability sets in for values of $\beta > 30^\circ$. This limitation of β has relatively little influence on Δp when θ is greater than 90° , so that curve 6 offers a good approximation to the observed behaviour throughout.

(ii) Rigidity of hair pile

As has already been shown the resistance of the hair pile to collapse is a most important factor which must be taken into account in considering the mode of

action of the plastron. It is possible to calculate the order of magnitude of the forces involved when external pressure is applied to the hair pile if a reasonable value for Y (Young's modulus) of the epicuticular hairs can be assumed. From the evidence discussed above we think it justifiable to assume that the hairs include in their make-up a highly cross-linked protein framework with perhaps a molecular orientation along the axis.

Values of Y for a series of substances of polymeric character likely to be of the same order are shown in Table 1.

Table 1

(Based on figures in 'Handbook of Chemistry and Physics', Chem. Rubber Publ. Co., Cleveland, Ohio.)

Plastic	Young's modulus (dynes/sq.cm.)	Plastic	Young's modulus (dynes/sq.cm.)
Cellulose acetate	0.2×10^{11}	Phenol formaldehydes:	
Cellulose nitrate	0.2×10^{11}	Good quality	1.4×10^{11}
Methyl methacrylate	0.35×10^{11}	Poor quality	0.4×10^{11}
Polyvinyl-chloracetate	0.3×10^{11}	Urea formaldehyde	1.0×10^{11}
		Phenolic-furfural:	
		Mineral filled	3.0×10^{11}
		Good quality	2.0×10^{11}
		Poor quality	0.7×10^{11}
		Wood (average)	1.0×10^{11}

This table indicates that, while amorphous linear plastics have a low Young's modulus of about $0.2-0.4 \times 10^{11}$ dynes/sq.cm., cross-linked plastics containing the CO.NH bond are much stronger and have values of the order of 1.0×10^{11} . Wood, which contains orientated crystallites of cellulose and some cross-linking, is of the same order; if allowance is made for the voids, the strength would be about 50% greater. These figures suggest that for a well cross-linked protein framework the modulus would be in the order of $0.5-1.0 \times 10^{11}$ dynes/sq.cm., and possibly a little higher if the chains are orientated.

We shall use this value as an approximate order of magnitude. The length of the hairs is $h = 5 \times 10^{-4}$ cm., and the radius = 1.0×10^{-5} cm. The latter figure introduces the largest error since it is raised to the fourth power. In all the insects studied where the hairs are easily visible (e.g. *Haemonia*, sense organ in *Aphelocheirus*, *Elmis*) there is a slight widening at the base of the hair giving greater rigidity; hence $r = 1.0$ will be a minimum value.

To calculate resistance to compression two methods were employed:

(a) Euler's treatment. The hair is regarded as a flexible rod subjected to compressional force causing buckling. When the applied force is critical the rod bends to a stable position so that

$$F = YAK^2 \frac{\pi^2}{h^3}, \quad (5)$$

where F = critical force, Y = Young's modulus, A = area of cross-section, K = radius of gyration of cross-section about neutral filament, and h = length.

For a cylindrical rod $K^2 = \frac{1}{4}r^2$, and for F we may put $\Delta P/n$, assuming equal distribution of the pressure over n hairs/sq.cm. ($n = 2 - 3 \times 10^8$). Therefore

$$\Delta P = 2 \times 10^8 \times \pi \times 1 \times 10^{-10} \times \frac{1 \times 10^{-10}}{4} \frac{\pi^2}{25 \times 10^8} \left\{ \frac{1.0}{0.5} \times 10^{11} \right. \\ \left. = \left\{ \begin{array}{l} 6.2 \\ 3.1 \end{array} \right\} \times 10^6 \text{ dynes/sq.cm.} \rightleftharpoons \left\{ \begin{array}{l} 6 \\ 3 \end{array} \right\} \text{ atm.}$$

(b) Treat the hair as a rod with distributed weight, the weight being

$$W = \Delta P/nh \text{ dynes/cm.}$$

Calculation indicates the critical condition of stability is where

$$\frac{Wh^3}{AK^2} = 7.84 \quad (6)$$

(Champion & Davey, 1936). Hence

$$\Delta P = 7.84 YAK^2n/h^3 \quad (7)$$

$$= 7.84 \times 1 \times 10^{11} \pi \times 10^{-10} \frac{10^{-10}}{4} 2 \times 10^8 / 25 \times 10^{-8} \\ = \left\{ \begin{array}{l} 4.9 \\ 2.5 \end{array} \right\} \times 10^6 \text{ dynes/sq.cm.} \rightleftharpoons \left\{ \begin{array}{l} 5.0 \\ 2.5 \end{array} \right\} \text{ atm.}$$

There is reasonable agreement between the two calculations.

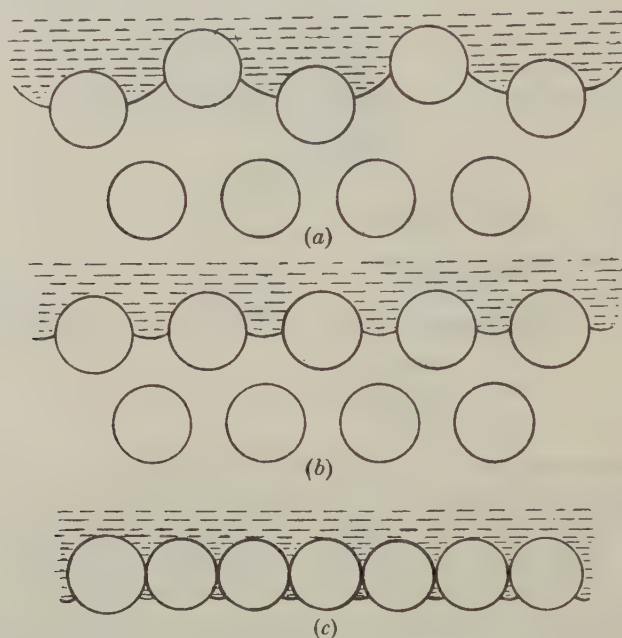
These values indicate that the critical applied pressure at which the hairs will bend is probably about 2.5–6 atm. This is very close to that at which wetting occurs in clean water. However, as the hairs are bent down under pressure, they will eventually lie over one another, since the area of the hair lying flat is $5 \times 0.2\mu^2 = 1\mu^2$, which is greater than the area allowed per hair, $0.3\text{--}0.5\mu^2$. In this condition they will not only be able to exert a much greater mechanical resistance to compression, but their proximity will increase the resistance to wetting (vide pp. 261, 264–5). But owing to the squeezing out of most of the air, the plastron will no longer be able to function as a gill and may not show the typical sheen.

Evidence that bending of the hairs did actually occur was obtained during pressure experiments. When pure distilled water was employed, the sheen did not darken completely, but 'flashed' darker on the initial stroke of the pump at about 2.5 atm. excess pressure. At 3.7 atm. it remained dark all over but did not acquire the dull black colour of specimens wetted with butyl alcohol. Moreover, immediately on release of pressure the sheen partially returned. With 0.5% butyl alcohol ($\theta = 106\text{--}107^\circ$) the phenomenon of 'flashing' just preceded wetting and the return of sheen was only just visible.

It is also interesting to note that when darkening occurred on account of the hairs bending over, this took place first in the mid-line of the sterna where the hairs are longest, and only at a higher pressure at the lateral borders. Such would be expected, since it will be seen from equations (5) and (7) above that Δp is proportional to h^{-2} , where h is the length of the individual hairs.

(iii) *Waterproofing effect of adpressed hairs*

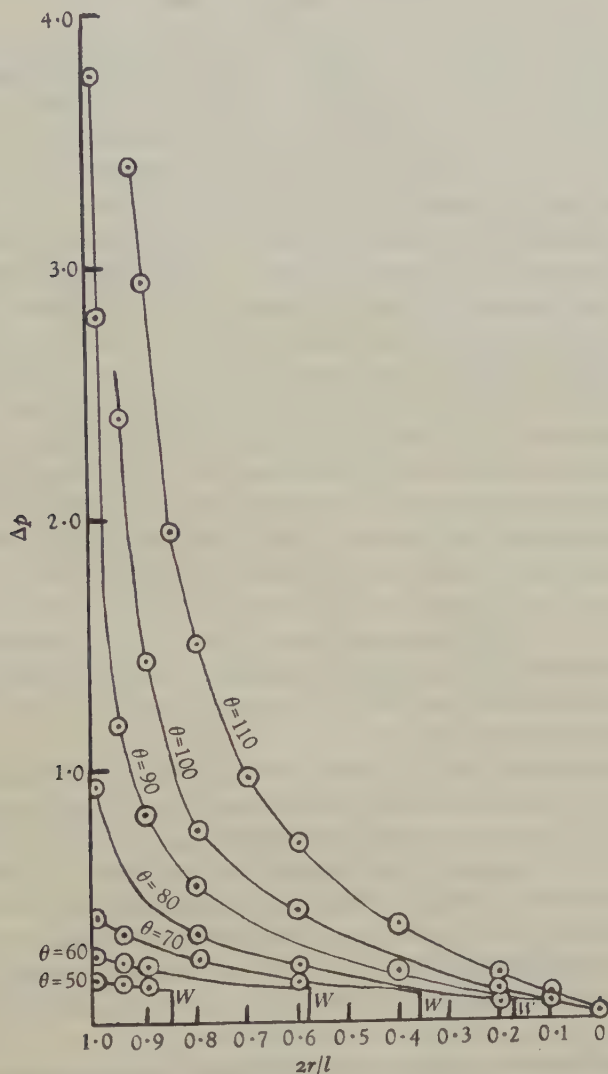
The pair of sense organs which lie on the outer posterior border of the second abdominal (apparent first abdominal) sternum admirably illustrate the above-mentioned waterproofing properties of closely packed adpressed hairs. Their size is about ten times that of the body hairs, they are very long (Text-fig. 27), and lie parallel to one another so that they overlap considerably.



Text-fig. 27 (a), (b) and (c). Diagram showing tendency of hairs to concentrate at an interface. (For explanation see text.)

Where this hair pile is in contact with the water surface the hairs, if free to do so, will tend to concentrate at the interface (Text-fig. 27*a, b*), since in this way the area of free water surface is reduced. In the limit the hairs will be touching each other at their diameters (Text-fig. 27*c*); this will take place if a downward pressure is applied to the interface to bring a sufficient number of hairs into it. In this system the equations developed for a horizontal array of hairs may be employed (equations (3) and (4)), but instead of a fixed distance l between adjacent hairs, the distance l will be gradually reduced to a limiting value of $2r$ as the hairs become concentrated at the interface. In Text-fig. 28 the maximum excess pressure Δp above such a system is plotted as a function of the distance separating the hairs. Δp is expressed in atmospheres and the dimension of the hairs is taken as 1×10^{-4} cm. radius as found in the sense organ. The values of γ are related to θ as in Text-figs. 24 and 25, being based on measurements of contact angles of butyl alcohol on wax blocks. It will be seen that for contact angles greater than 90° there is no limit to the value of Δp ,

provided the hairs are sufficiently close; for smaller contact angles Δp has a maximum value when the hairs are in contact, which is of the order of 1 to a small fraction of an atmosphere, but always positive. As will appear from a succeeding paper,



Text-fig. 28. Curves showing the maximum excess pressure (Δp in atmospheres) sustained by systems of horizontally arranged adpressed hairs of the size found on the pressure sense organ of *Aphelocheirus* for different degrees of packing and different contact angles. Points marked *W* indicate where the system breaks down since $\beta > 90^\circ$ and hairs are completely wetted. Degree of packing expressed as ratio $2r/l$.

although the hairs are much larger and on that account present relatively smaller surface forces, the close packing will account for their greater resistance to wetting. This resistance should be reduced sharply when θ falls below 90° . If the hairs were of the same radius as the body hairs the magnitude of Δp would be ten times larger;

but, although well waterproofed, the air-water surface would be so reduced that the structure would not function as a respiratory organ.

6. SUMMARY

1. As is well known from the work of Ege and others many aquatic insects (e.g. Dytiscidae, Hydrophilidae, Corixidae, Notonectidae) which carry air stores in the form of bubbles or films attached to their bodies or in special subelytral spaces can, if the water is well aerated, obtain a proportion of their total oxygen requirement by diffusion across the air-water interface of the exposed bubble surface just as if it was a gill. These insects, however, have no means of preventing decrease in the volume of their bubbles; consequently if they remain submerged for too long the tension difference which is the basis for the inward diffusion of oxygen will also bring about a slower outward diffusion of nitrogen, with the resulting danger of loss of bubble and final waterlogging. Nevertheless, in contrast to the vast majority of adult aquatic insects which carry substantial 'air-stores' with them in the form of bubbles or thick films or in special subelytral chambers, there are certain insects which have evolved a technique of holding an extremely thin film of gas, of negligible volume, on the surface of their bodies by means of the surface forces provided by a system of minute hydrofuge hairs. This type of air film we call the 'plastron'. It differs fundamentally from any kind of 'air store' in that the surface forces involved are of sufficient magnitude to hold the interface in position, effectively resisting any changes in pressure and surface tension of the medium that the insects are likely to meet, and so maintaining a *constant volume* of gas. Such a plastron acts solely as a gill, and insects which possess it can stay permanently below the surface and, as long as the medium is sufficiently well aerated, thus become virtually independent of contact with atmospheric air.

2. For various reasons the only British insect which is in practice a suitable experimental animal for the study of plastron respiration is the Hemiptera *Aphelocheirus aestivalis*. It is mainly an active predatory insect, inhabiting rapidly flowing streams and rivers. It appears to have its main habitat in the large rivers of eastern Europe and Scandinavia, and to be very scarce in other parts of Europe. In Britain it has probably been largely overlooked and may in fact be commoner than has been supposed. The wingless form is the only one known in this country. An outline of its life history and ecology is given. The nymph has the tracheal system closed and lacks the plastron, gas exchange being entirely cutaneous.

3. The greater part of the body surface of the adult *Aphelocheirus* is covered with an extremely fine plastron held in position by an epicuticular hair pile having approximately 2,000,000 hairs per sq.mm. The structure and dimensions of the plastron hairs have been investigated by means of ultra-violet photography and by the electron microscope. The tracheal system is described in detail; its chief characteristic consists in the greatly modified abdominal spiracles which take the form of 'rosettes' of branching tubes in the exocuticle, filled with plastron hairs and opening by numerous minute pores into the plastron of the ventral surface. This modification of the spiracles gives a highly efficient protection against the entry of water.

4. Experiments have shown that, provided the water is kept well aerated, no contact with the atmosphere is necessary; nor, indeed, are visits to the surface of much use since there is no provision for carrying any air store. It is shown that the gaseous plastron is retained intact and the hairs unwetted even when the animal is submerged in oxygen-saturated water (cf. the 'Ege experiment') or in gas-free water. The respiratory function of the different regions of the plastron has been studied by experiments in which a given part or the whole of the gaseous plastron has been removed by treatment with a suitable wetting agent. The behaviour of such animals is then observed when they are kept in waters of different known tensions of oxygen. The oxygen deficiency tolerance of nymphs was also investigated. It appears that in the nymph, in which the flattened lateral margins of the abdominal segments are specialized to some degree as tracheal gills, the body surface/volume ratio is such that simple diffusion of oxygen through the cuticle is adequate to supply all the needs of the animal. When, however, the insect reaches the size characteristic of the late fifth instar, a critical point is reached at which the diffusion across the cuticle is no longer sufficient, and a new method of respiration, the plastron, becomes a necessity. This subject will be considered in detail in a succeeding paper.

5. The plastron hairs are epicuticular, but when sections are examined by ultra-violet photomicrography it is seen that the hairs possess root-like structures which pass through several layers of exocuticle, layers which are indistinguishable by other methods. Pore canals can be seen in the endocuticle but not certainly in the exocuticle.

6. The resistance of the hair pile to wetting by surface forces was determined by treatment with graded concentrations of pure isobutyl alcohol, the replacement of sheen by a dull black colour being the indication that wetting had taken place. Wetting proceeds slowly in 10% butyl alcohol and rapidly at 12% (contact angle 65° , surface tension 26 dynes/cm.). The sheen cannot be restored by immersion in water supersaturated with air at atmospheric pressure, but if the cuticle is thoroughly washed and then dried in air, resistance to wetting is again practically normal.

7. The resistance to wetting by pure water under increased hydrostatic pressure was studied. It was found that the sheen disappeared at excess pressures between 3.5 and 5.0 atm. In this case the blackening is not as complete as on wetting with butyl alcohol and a considerable recovery takes place when pressure is released—evidence that the darkening in this case is due not to wetting but to a collapse of the hair pile itself. In discussing the rigidity of the hair pile, it is shown that a reasonable agreement between theory and observation exists if it is assumed that the material of which the plastron hairs are composed has a Young's modulus of $0.5-1.0 \times 10^{11}$ dynes/sq.cm. This figure is considered to be a reasonable one in view of the available information on the chemical nature of the hairs and the Young's modulus of a variety of 'plastic' and other substances. Wetting by a graded series of butyl alcohol solutions of less than 10% under increased pressure was also studied.

8. A mechanical theory is formulated for the resistance of a system of hydrofuge hairs arranged in various ways. It is shown that the structure, dimensions and packing of the plastron hairs are such as to give what must be nearly the most favourable

compromise between the conflicting requirements of high resistance to wetting and to mechanical collapse on the one hand and of a large water-gas interface for respiratory exchange on the other. The system of larger recumbent hydrofuge hairs found only on the organs of pressure sense, while even more resistant than the general plastron to wetting, is shown to suffer from serious theoretical disadvantages as a respiratory structure.

It is a pleasure to acknowledge our indebtedness to Mr E. A. Ellis, Naturalist at the Norwich Museum, for generous assistance and advice in discovering localities for *Aphelocheirus* and *Haemoma*. Similarly, we are indebted to Lt. E. S. Brown for much valuable information as to the behaviour and habitat of *Aphelocheirus* in the River Cherwell. To the authorities and staff of the Mount Vernon (Hampstead) Laboratory of the Medical Research Council and of the Cavendish Laboratory, Cambridge, we are indebted for unstinted help and facilities in the use respectively of the ultra-violet photomicrograph and the electron microscope. Finally, we are greatly indebted to Prof. E. K. Rideal, F.R.S., for first suggesting that D.J.C. should collaborate in this investigation.

REFERENCES

- BEDWELL, E. C. (1945) (ed. A. M. Massee). *Ent. Mon. Mag.* **81**, 253.
 BELING, D. (1926). *Mém. Cl. Sci. phys. math. Kiew*, **4**, part 4.
 BIKERMAN, J. J. (1940). *Trans. Faraday Soc.* **36**, 412.
 BROCHER, F. (1912a). *Ann. Biol. lacust.* **5**, 5.
 BROCHER, F. (1912b). *Ann. Biol. lacust.* **5**, 136.
 CASSIE, A. B. D. & BAXTER, S. (1944). *Trans. Faraday Soc.* **40**, 546.
 CHAMPION, F. L. & DAVEY, N. (1936). *Properties of Matter*, **3**, 56-9. London: Blackie and Son.
 COMSTOCK, J. H. (1887). *Amer. Nat.* **21**, 577.
 DECKSBACH, N. K. (1921). *Mem. Soc. Nat. Jaroslaw*, **3**.
 DECKSBACH, N. K. (1923). *Arb. biol. Wolga-Sta.* **6**, 281.
 DENNELL, R. (1946). *Proc. Roy. Soc. B*, **133**, 348.
 EGE, R. (1918). *Z. allg. Physiol.* **17**, 81.
 HARKINS & JORDAN (1930). *J. Amer. Chem. Soc.* p. 1751.
 HURST, H. (1945). *Brit. Med. Bull.* **3**, 132.
 KULMATYCKI, W. & GABANSKI, J. (1928). *Arch. Hydrobiol. Rybact.* **3**, 111.
 LARSEN, O. (1924). *Ark. Zool.* **16**, no. 16, pp. 1-21.
 LARSEN, O. (1927). *Ent. Tidskr.* **48**, 181.
 LARSEN, O. (1932). *Int. Rev. Hydrobiol.* **26**, 1.
 MAPLE, J. D. (1937). *Ann. Ent. Soc. Amer.* **30**, 123.
 PEARCE, E. J. (1945). *Ent. Mon. Mag.* **81**, 139.
 ROYER, M. (1913). *Bull. Soc. Ent. Fr.* pp. 243-7.
 SCHAUFUSS, C. (1916). *Calwer's Kaferbuch*, **1**. Stuttgart.
 SIROTININA, O. (1921). *Arb. biol. Wolga-Sta.* **5**, 335.
 SZABO-PATAY, J. (1924). *Ann. Mus. Nat. Hungary*, **21**, 33.
 THORPE, W. H. (1936). *Parasitology*, **28**, 517.
 USSING, H. (1910). *Int. Rev. Hydrobiol.* **3**, 115.
 WEBER, H. (1930). *Biologie der Hemipteren*. Berlin: Springer.
 WENZEL, R. N. (1936). *Industr. Engng Chem.* **28**, 988.

EXPLANATION OF PLATES

PLATE 6

Fig. *a*. Sections showing plastron of abdominal sternum of adult *Aphelocheirus*. Sections cut with freezing microtome, mounted unstained in glycerine and photographed with ultra-violet radiation. Aperture 5 mm., exposure 5 sec. Note that with this exposure only the hair pile is visible, the epicuticle and exocuticle being impervious to the radiation and appearing completely black.

Figs. *b*, *c* and *d*. Same section as *a* with much greater exposure to show structure of epicuticle and exocuticle.

b. Note that the plastron hair pile is now so over-exposed as to be invisible, but the 'roots' of the hairs can now be seen passing through five of six separate lamellae which appear to constitute the epicuticle. The exocuticle is still almost completely opaque. Exposure 30 sec., aperture 5 mm.

c. Exposure 1 min. Note that the exocuticle is now partly transparent and that the 'roots' of the hairs continue somewhat obliquely through it.

d. Exposure 2 min. The 'roots' can now be seen passing right through the exocuticle and disappearing in the outer layers of the endocuticle. Magnification of *a-d* $\times 2850$.

(N.B. The arrow marks the same spot on the section in all four photographs.)

PLATE 7

Fig. *e*. Section through abdominal spiracular rosette of adult *Aphelocheirus*. Method as above. Aperture 5 mm., exposure 3 min. Magnification $\times 2850$.

Fig. *f*. Transverse section of sternal cuticle of fifth instar nymph of *Aphelocheirus*, method as above. Aperture 5 mm., exposure 2 min. Note absence of hair pile, simple epicuticle, dark exocuticle showing transverse line suggesting pore canals towards inner margin, and thick unpigmented endocuticle in which a large number of lamellae are faintly visible. Magnification $\times 2850$.

Fig. *g*. Electron microscope photograph of gelatin-formvar preparation of plastron surface of abdominal sternum of adult *Aphelocheirus*. The numerous small somewhat triangular bodies represent the tips of the plastron hairs. Magnification $\times 91,200$.

Fig. *h*. Electron microscope 'silhouette' of a small part of a section of abdominal sternum showing tip of a single hair projecting from the edge of the hair pile which is itself quite opaque to the electron beam. Magnification $\times 120,000$.

STUDIES ON PLASTRON RESPIRATION

II. THE RESPIRATORY EFFICIENCY OF THE PLASTRON
IN *APHELOCHEIRUS*

BY W. H. THORPE

Sub-Department of Entomology, Zoological Department, University of Cambridge

AND D. J. CRISP

Department of Colloid Science, University of Cambridge

(Received 9 November 1946)

(With Ten Text-figures)

1. INTRODUCTION

In a previous paper (Thorpe & Crisp, 1947, later referred to as Part I) the plastron-bearing mechanism of *Aphelocheirus* was described in detail. The ability of this structure under good conditions to retain a layer of gas against a pressure deficiency of several atmospheres was demonstrated, and preliminary experiments were carried out in order to establish that the plastron was in fact respiratory in function. In this paper we submit quantitative evidence in support of this view, and show that efficient respiration is possible through the plastron by ordinary diffusion. A comparison is made between the efficiency of the cutaneous respiration of the fifth instar nymph and that of the plastron of the adult.

2. OXYGEN REQUIREMENTS OF *APHELOCHEIRUS*

The first step in this investigation was to obtain a figure for the basic respiratory rate, and if possible for the oxygen uptake in various states of activity. Owing to the small size of the animal micro-methods were more appropriate, but in the first instance a standard Barcroft manometer was employed. Table 1 gives the results obtained from experiments in which three or four bugs were used at a time.

Experiments were later carried out on individual animals, for which the ordinary Barcroft respirometer was not sufficiently sensitive. For these experiments a new apparatus was devised which operated on the same principle as the Barcroft manometer, but was about 100–200 times as sensitive, and will be described separately (Crisp & Thorpe, 1947). It will be designated a 'metal Barcroft micro-respirometer', as its most important feature is its construction from a metal block.

Results obtained using this instrument are given in Table 2 and are in general agreement with the figures obtained from the standard Barcroft manometer.

It will be observed from Tables 1 and 2 that the basal metabolic rate (when the insect is at rest) is about 6 cu.mm./hr. for the adult, and for the nymph about 3 cu.mm./hr., at a temperature of 20°C. This corresponds in each case to 150 c.c./hr./kg. live weight, which is rather low in comparison with figures given by Ege (1918) for some other aquatic insects. It will be noted, however, that very

Table 1. *Results using standard Barcroft apparatus. Experiments carried out October–November 1945*

Exp.	Condition of animals	Temp. ° C.	Uptake (cu.mm./ hr./individual)
1. Adults			
I 3 bugs	Very wet.* Sheen lost towards end of experiment. One died. 2 months after capture	19	3.6
II 3 bugs	Almost dry. Sheen reduced at end of experiment.* Age as above	19	5.85
III 3 bugs	Normal, inactive. Age as above	19	4.42
IV 3 bugs	Inactive. Sheen lost at end of experiment. Age as above	19	4.72
V 3 bugs	Normal at end. About 2½ months after capture	19	3.51
VI 4 bugs	Freshly collected. Rather active	18	7.55
2. Nymphs			
VII 5 bugs	Normal, rather active. 2 months after capture	18	3.1
VIII 4 bugs	Quiescent. 2½ months after capture	18	2.2

Weight of adults: 0.04 ± 0.003 g. Weight of nymphs: 0.02 ± 0.004 g.

* Loss of sheen found to be due to contamination by potash.

Table 2. *Results using metal Barcroft micro-respirometer on single individuals*

Date	Exp.	Condition of animals	Temp. ° C.	Uptake (cu.mm./ hr./individual)
1. Adults				
Dec. 1945	IX	2 weeks after capture. Normal	20	8.4
	X	Active	20	11.5
	XI	Normal	20	6.6
	XII	Sluggish	20	5.4
	XIII	Struggling violently	20	17–18
Jan. 1946		Quiescent or normally active	20	5.1–6.7
	XIV	1 week after capture, normal	20	9.1
	XV	1 week after capture, normal	20	6.2
	XVI	Active	20	14.4
	XVII	Well-fed individual, struggling continuously, 1 week after capture	20	32.0
Mar. 1946	XVIII	2 weeks after capture, moving	20	10.6
	XIX	2 weeks after capture, moving	20	12.8
	XX	Prolonged and continuous movement, well fed	20	23
2. Nymphs				
Dec. 1945	XXI	Quiet 6 weeks after collecting	20	1.7–2.4
	XXII	After treating with atmosphere of 4% oxygen content condition as above	20	3.4
	XXIII	After treating with atmosphere of 1% oxygen content condition as above	20	6.6
	XXIV	Normal	20	2.2
	XXV	Quiet after long period of low oxygen tension (4%)	20	2.5
Jan. 1946	XXVI	Very active 7 weeks after collecting	20	5.3
	XXVII	Very active, freshly collected	20	7.4
	XXVIII	Quiescent after long periods at low oxygen tension (5%)	20	4.9
Mar. 1946	XXIX	Spasmodically active, well fed	20	5.5
	XXX	Active, 2 weeks after collecting	20	5.55

considerable variations occur according to the condition of the insect, and the values given above are from selected experiments in which the animal remained quiet for long periods. Moreover, there is definite evidence that the respiratory rate was higher in freshly collected material, probably on account of the slow starvation of the animals under laboratory conditions where the normal food could not be provided in sufficient quantity. Since the animals often died after about 2 months in captivity, it is not unreasonable to attribute the loss of activity and slowing down in metabolic rate to this cause. It is possible, too, that a seasonal fluctuation occurs in the metabolic rate; though the evidence for this is scanty, the exceptionally high figures recorded from specimens taken early in March suggests that this was possibly the case.

No data on the influence of temperature on respiratory rate was considered necessary, as the maximum temperature likely to be met by the insect in nature is about 20° C.; lower temperatures reduce metabolism and favour oxygen uptake, so that this figure for the basal rate of metabolism is the maximum that need be considered.

The increase in uptake when the insect is active is clearly illustrated. Adults appear in general to be more active than nymphs, and when struggling violently the respiratory rate may rise six- or sevenfold. Owing to the confined space which the animals occupy during experiments their maximum activity is not reached, and it is probable that a strongly swimming adult would absorb more than six or seven times the basal oxygen uptake.

A small number of observations were carried out on adults under water, using a micro-Winkler technique similar to that described by Fox & Wingfield (1938). In our experiments it was necessary to reduce the scale of the micro-pipette somewhat, as the quantities for estimation were very small. Two or three insects were placed in 8–10 c.c. of water of known oxygen content under a gauze frame, and the surface of the water sealed with Nujol. Samples of 0.5 c.c. were withdrawn by a capillary siphon at intervals and taken up into an Aglar micro-syringe. 0.1 c.c. was first taken up, followed by 0.01 c.c. of manganese sulphate and 0.01 c.c. of Winkler's reagent. The pipette was then filled up to 0.5 c.c. with the rest of the solution and left standing for 10 min. with occasional shaking. 0.02 c.c. of 50% H_2SO_4 was finally drawn in and mixed till all the precipitate dissolved, and the resulting solution titrated against $N/200$ thiosulphate as described below. The order of introducing reagents into the micro-syringe was important in order to obtain a flocculent precipitate of manganous hydroxide.

In titrating the iodine liberated against thiosulphate, two difficulties not mentioned by Fox & Wingfield were encountered. First, we found that a metal syringe needle must be avoided as it rapidly caused reduction of iodine to iodide. Secondly, we found that very careful control observations were necessary in order to obtain a reliable starch-iodine end-point, owing to the failure of starch to acquire a blue colour until a definite excess of iodine had been added. This phenomenon is not important when the total quantity of iodine being estimated is large, but with quantities of the order of 10^{-5} g. the error is of the order of 50% or more. The

following procedure was found to be reliable. A 1% starch solution is diluted 20 times and $N/1000$ iodine solution run into it until it changes from yellowish brown by transmitted light to a clear or faintly opalescent blue-grey tint. About 0.3 c.c. of this is placed in each of a row of four 0.5 c.c. tubes, and the iodine to be estimated is run into each from the Aglar syringe, 0.005 into the first, 0.01, 0.015 and 0.02 c.c. into the others. These are control tints indicating the colour when the iodine has 'overshot' by varying amounts. To the last tube of starch 0.05 c.c. of $N/200$ thiosulphate is added from a micro-syringe and the iodine solution added to this until a blue tint appears. This is matched successively with each of the control tubes in order to determine the exact quantity of iodine for neutralization. It is important to make up controls just prior to conducting a titration, as they rapidly deteriorate; they should also be kept at the same temperature as the experimental tube as the intensity of colour appears to be somewhat temperature-dependent.*

These observations gave an average value of 5.5 cu.mm. O_2 /adult/hr., in agreement with the foregoing. Unfortunately, the Nujol seal was found to be unsatisfactory, differences being obtained according to the oxygen content of the Nujol. The figure above was obtained as a mean between observations using air-saturated and oxygen-free Nujol, but it was afterwards considered advisable in all such experiments to employ mercury only as a seal owing to the solubility of oxygen in organic liquids.

3. DIFFUSION RESISTANCE AS A MEASURE OF RESPIRATORY EFFICIENCY

In Part I a number of qualitative experiments are described which indicate that the plastron is efficient as a respiratory organ, but no measure of its relative efficiency compared with cuticular respiration is possible from behaviour records.

Owing to the very flattened shape of *Aphelocheirus* it is not possible to employ the technique of biological indicators (Thorpe, 1932) to show the relative importance of the various sites of oxygen uptake. It is necessary, therefore, to employ indirect means of assessing the value of the plastron.

A measure of respiratory efficiency which is independent of the momentary rate of metabolism is required, and for this the concept of diffusion resistance is most suitably employed.

If through any closed path of diffusion† there exists a fall in tension of the gas of Δp atmospheres, and the quantity flowing is q units/sec., then the diffusion resistance of this path is

$$R = \frac{\Delta p}{q} \quad (1)$$

Thus, if the path considered is the over-all uptake from the surrounding medium

* These complications of method will perhaps have been rendered unnecessary by the recent development of sodium starch glycollate as an indicator for iodometric analysis. (See Peat, S., Bourne, E. J. & Thrower, R. D. *Nature*, 159, 810-11, 14 June 1947.)

† A path of diffusion in which no diffusing material enters or leaves the system other than at the origin and termination.

into the tissues, Δp will be the difference in tension between the outer medium and the mean internal tension, while q will be the rate of uptake of oxygen by the tissues. It is sometimes necessary to compare the respiratory efficiency of one organism with another, and for this purpose diffusion resistance alone is not sufficient, because their normal requirements may differ. The most suitable index of oxygen requirement would appear to be the basal metabolic rate q_b , and as a measure of respiratory efficiency we shall introduce a quantity, hereafter referred to as the 'respiratory index' of $1/q_b R$. The units of this quantity will be (atm.)⁻¹, and it represents simply the reciprocal of smallest tension difference between internal and external media compatible with maintaining basal metabolism.

In general the diffusion of oxygen into the animal may be divided into a number of elementary paths of diffusion of which some may be in series (as, for instance, the different cuticular layers bounding the external surface), and others in parallel (as, for instance, a row of independently opening spiracles). The well-known equations for equivalent resistances may then be employed if it is desired to calculate the over-all resistance from a number of elementary ones:

$$R_{\text{series}} = R_1 + R_2 + R_3, \quad (2)$$

$$R_{\text{parallel}} = 1 / \left(\frac{1}{R_1} + \frac{1}{R_2} + \frac{1}{R_3} \text{ etc.} \right). \quad (3)$$

4. MEASUREMENT OF OVER-ALL DIFFUSION RESISTANCE

The most direct method is to measure Δp and q directly in equation (1). The measurement of the internal oxygen tension may be achieved by Krogh's method (Krogh, 1908), in which a bubble of air is introduced into the animal tissues, and subsequently squeezed out and analysed after time has been allowed for equilibration.

This method was not considered suitable in the case of a very much flattened insect such as *Aphelocheirus*, since

(a) A bubble of size sufficient for analysis would interfere with the normal behaviour.

(b) It would be impossible to confine such a bubble to the tissue fluids without contact with the tracheal vessels or cuticle.

(c) The introduction of the bubble presents considerable difficulty.

The alternative method employed here is to measure the oxygen uptake of the animal when exposed to successively lower tensions of oxygen. The horizontal axis in Fig. 1 represents oxygen tension, the vertical represents rate of uptake of oxygen (q). Fig. 1 *a* represents the normal condition when the surroundings contain 20% oxygen; the tissues will not be at uniform tension but will have a distribution as represented by the small hump, at mean tension Δp below the surroundings. The uptake is indicated by the point *A*. As the external tension is lowered the internal tension will be reduced correspondingly, as is shown in Fig. 1 *b*. Eventually, however, the oxygen tension in the tissues will begin to reach a limiting threshold (marked *P*) below which the tissues cannot make use of oxygen and below which their oxygen tension will therefore never fall. As this happens q begins to fall off

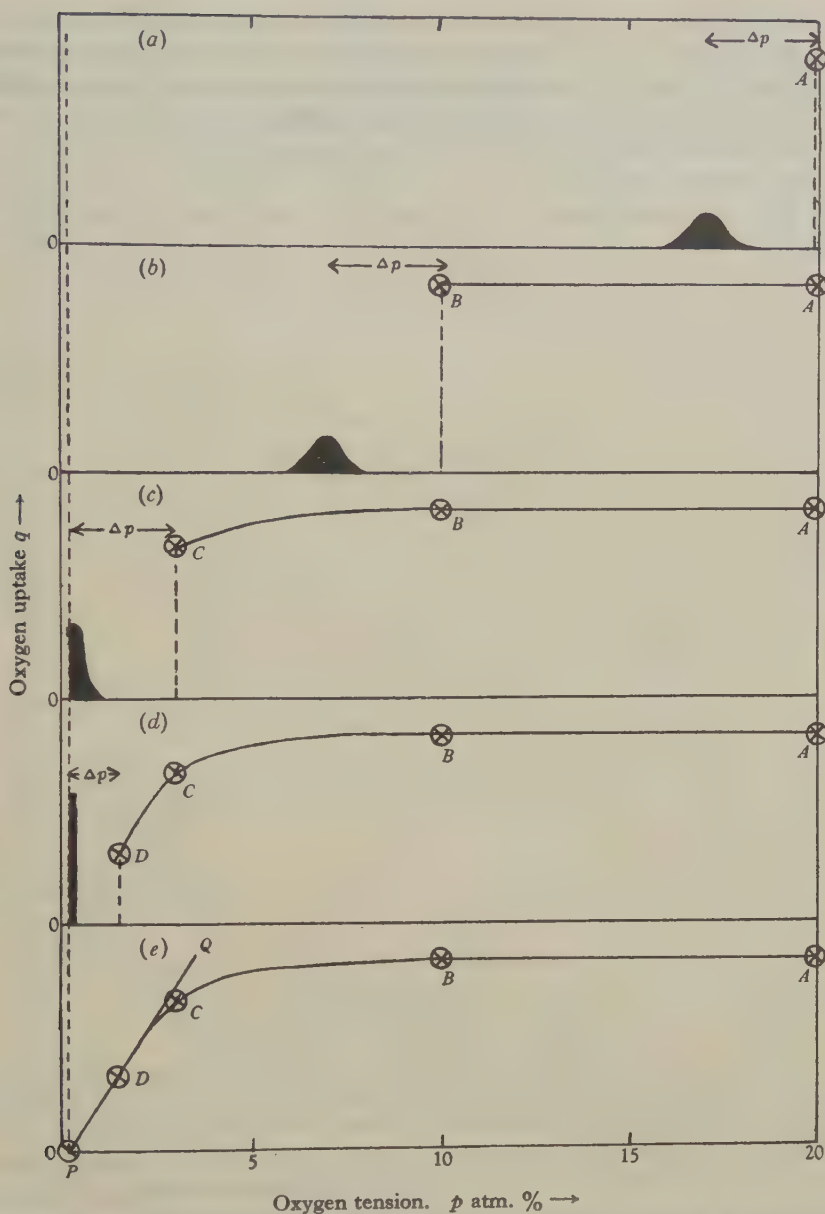


Fig. 1. Schematic representation of oxygen uptake of an organism exposed to diminishing oxygen tension. P represents the threshold oxygen tension for tissue respiration. Δp indicates the mean tension difference between surroundings and the tissues, the shaded area showing the distribution of oxygen tension in the tissues. $\otimes$ Points thus shown represent oxygen uptake at a given external oxygen tension.

(Fig. 1 c). When all the tissues are at threshold tension (Fig. 1 d) $\Delta p = p - P$ and q is limited entirely by the rate of diffusion of oxygen in the system, also determined by $p - P$, so that in this region the relation between p and q is represented by a

straight line (PQ , Fig. 1 *e*). The relation between q and Δp is also obtainable from this straight line, and from it the diffusion resistance $R = \Delta p/q$ can be calculated. In all our observations the threshold internal tension is not appreciably different from zero, so that the line PQ appears to cut the origin. This implies that the tissues

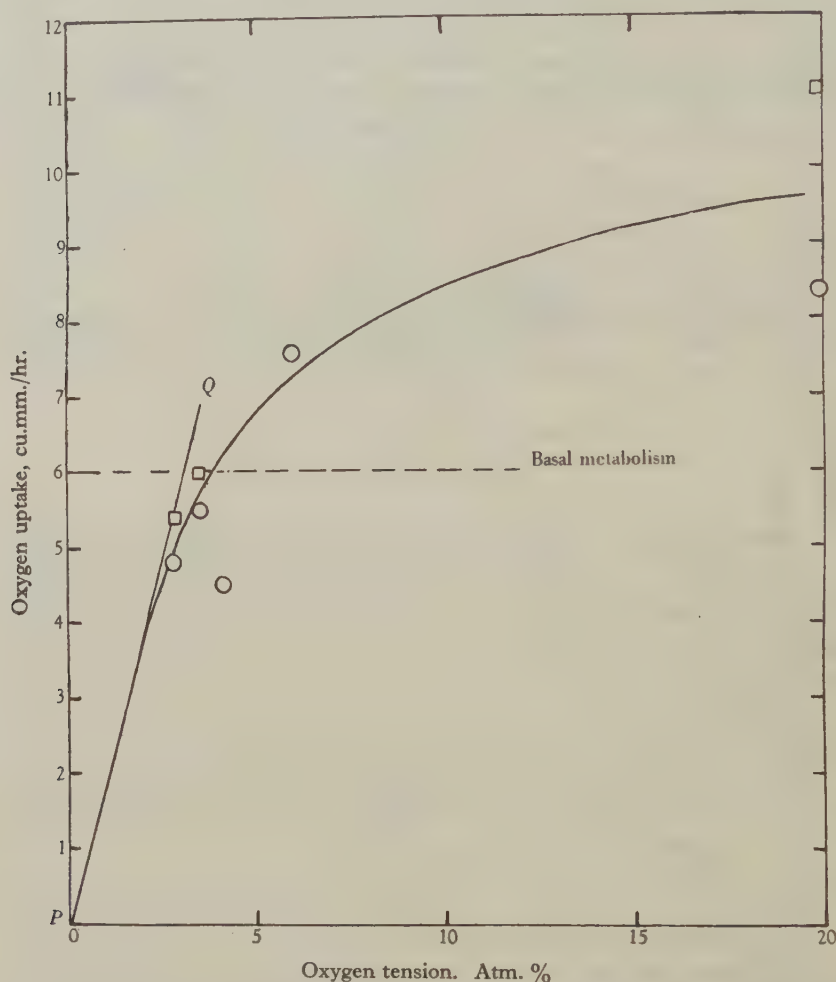


Fig. 2 A. Oxygen uptake of adult *Aphelocheirus* using modified glass Barcroft apparatus with small vessel. Larger vessel uptake 1.17h, smaller vessel uptake 1.05h.

are capable of abstracting oxygen at very low tensions indeed, in agreement with observations by Maloeuf (1936). However, it is unlikely that tissues deprived severely of their normal oxygen supply will escape uninjured, and this constitutes the chief weakness of the method. Not only are the vital activities and enzyme systems likely to be damaged by prolonged exposure to low oxygen tensions, it is also possible for the animal to go into debt for oxygen and increase its rate of uptake when the external tension is raised. For this reason we have endeavoured to obtain the $q-p$ curves while the oxygen tension is being reduced, but not to maintain the

animal at low tensions longer than is necessary. The order in which observations were made is important and is given in the results.

Fig. 2 A shows the oxygen uptake of adult *Aphelocheirus* at varying oxygen tensions. These experiments were carried out in a small glass Barcroft apparatus which was not very sensitive and required rather a long period to obtain a satisfactory respiration rate. Consequently exposure to very low tensions was not possible, and the critical region where uptake is proportional to external oxygen tension is scarcely represented. The line *PQ* from which the diffusion resistance *R* is calculated gives

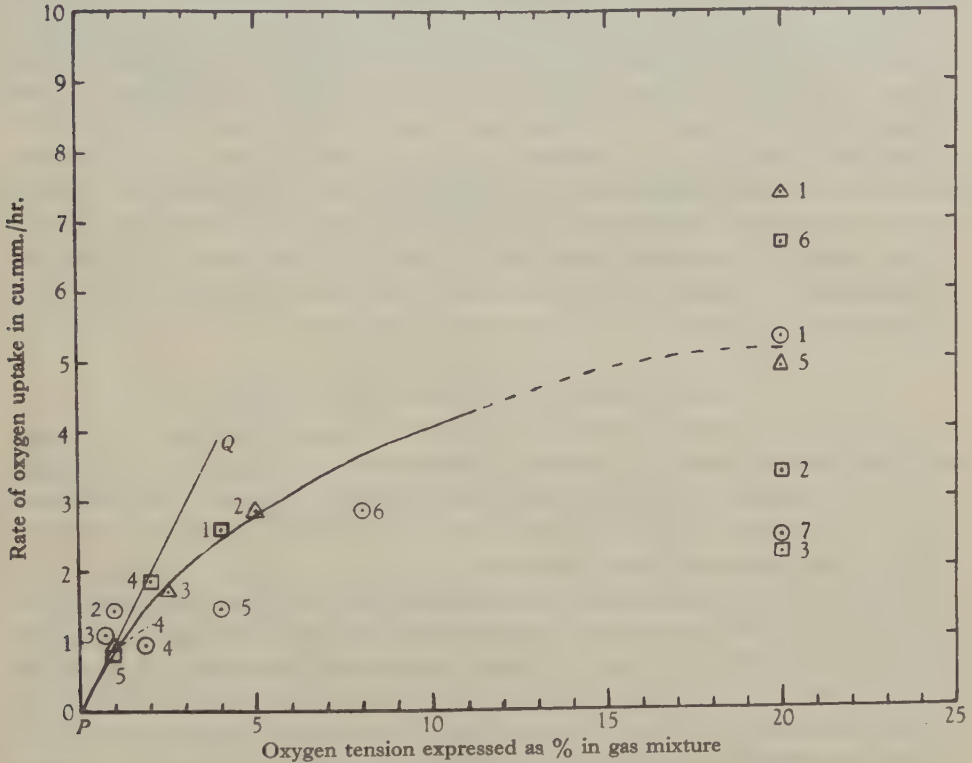


Fig. 2 B. Oxygen uptake of fifth instar nymphs of *Aphelocheirus*. $\square$ = specimen A: wt. 0.13 g.; dimensions 0.75 $\times$ 0.55 cm. $\odot$ = specimen B: wt. 0.25 g.; dimensions 0.75 $\times$ 0.60 cm. $\triangle$ = specimen C: wt. 0.22 g.; dimensions 0.80 $\times$ 0.65 cm. Numbers indicate order of successive observations.

therefore the maximum value for *R*. This is seen to be $R = 5.2 \times 10^{-3}$. When expressed in units represented on the graph (cu.mm./hr. and atm.) or expressed in more absolute units (c.c./sec. and atm.) the value is 1.9×10^{-4} . The respiratory index can be seen from the graph to be at least $1/0.031 = 32$.

Experiments on fifth instar nymphs shown in Fig. 2 B were conducted in a micro-respirometer (separately described) and are more satisfactory than the preceding, as it was possible to employ lower oxygen tensions for shorter periods. Moreover, it is possible to observe from these results some of the complications of this method.

Specimen A was maintained at 4% tension for 1 hr. and returned to normal air at 20%. There was initially a rise in uptake shown by point (2) due to the lower internal tension at first, but later the uptake dropped to the point marked (3). Exposure to 2 and 1% oxygen for periods of $2\frac{1}{2}$ and 1 hr. respectively did not permanently injure this animal, which on returning to 20% mixture had a much enhanced uptake (point 6).

Specimen B was initially very active with a rather high uptake, and was maintained at 1% for $2\frac{1}{2}$ hr. (points 2 and 3). When the oxygen tension was raised, however, recovery was slow and the animal appeared dead (points 4 and 5), until placed in 8% oxygen, when it partially recovered (point 6). In this case it appeared that some damage was caused by prolonged exposure to low oxygen tension, and this was confirmed by the animal failing to recover its initial activity (point 7) and adopting an asphyxia posture for some time after replacing in the aquarium.

Specimen C was the most satisfactory. It was initially very active, and was maintained at the low tensions $2\frac{1}{2}$ and 1% for only $1\frac{1}{4}$ and $\frac{3}{4}$ hr. respectively; the observations were made with progressively less oxygen present, and recovery was good.

From these three sets of results the line *PQ* has been constructed. This gives an over-all diffusion resistance of 3.7×10^4 abs. units, and a respiration index of 33 not measurably different from that obtained for the adult.

5. RESPIRATION THROUGH THE CUTICLE

In the fifth instar nymph respiration is wholly cuticular, whereas in the adult respiration will take place simultaneously through the cuticle and via the plastron, rosettes and tracheal system. It is therefore desirable to find out what fraction of the uptake in the adult travels by each route, and how the cuticle permeability of the nymph compares with that of the adult.

Attempts made to measure cuticle permeability directly were not successful owing to the great difficulty experienced in completely sealing a small area of cuticle without causing damage. However, it was found possible to obtain information on the cuticle permeability by means of experiments on oxygen uptake *in vivo*.

If it is assumed that in the limiting region where oxygen uptake is directly proportional to external oxygen tension, the tissues are approximately at zero tension, then the diffusion resistance measured represents the resistance of the whole cuticle. This assumption is a reasonable one, since the internal tissues are likely to be at a fairly uniform tension, but since there may be a small diffusion resistance in addition to that of the cuticle (e.g. the hypodermis) the value obtained for cuticle permeability obtained will be a minimum value. The area of the nymphal cuticle is 75 sq.mm., and the diffusion resistance 3.7×10^4 , giving a value for permeability of 3.6×10^{-5} c.c. O₂ at N.T.P./sec./atm. pressure difference/sq.cm. area.

In order to obtain the permeability of the adult cuticle in a similar manner, it is necessary to prevent any uptake by the spiracles. This was most conveniently arranged by covering the whole of the ventral surface of the animal with apeizon low melting-point wax. This wax can be melted at a temperature which is not harmful to the bug and sets firmly. The use of soft wax or vaseline as a seal was

unsatisfactory owing to the strength of the third thoracic limbs and of the thoracic muscles, which eventually freed the thoracic grooves and allowed tracheal respiration to recommence.

The uptake of sealed adults even in 20% oxygen was always considerably below the basal respiratory rate; the tissues could therefore be assumed to be at zero tension. After each experiment the area of dorsal surface exposed to the atmosphere was measured by camera lucida drawings, and the permeability calculated. The results, shown in Table 3, exhibit considerable variation, as might be expected when different portions of the terga are exposed, but some of the higher values may be due to incomplete sealing and leakage of air into the plastron. It will be seen, however, that the permeability is considerably lower than that of the nymph.

Table 3

Area of tergum exposed (sq.mm.)	Uptake (cu.mm./hr. at 0.2 atm. O ₂)	Permeability (c.c. at N.T.P./sec./sq.cm./atm.)
25	0.7	0.39×10^{-5}
25	0.3	0.72
26	1.15	0.62
36	1.66	0.64
20	0.7	0.49
9.3	0.5	0.75
20	1.3	0.90
23	3.5	2.1
58	3.2	0.76
45	2.7	0.83
60	5.1	1.2

Mean value: 0.85×10^{-5} c.c./sec./sq.cm./atm.

It was not possible to make observations on the permeability of the ventral surface, but its thickness and appearance in section were not in any way different from that of the dorsal, and it is unlikely that its permeability would be significantly different. The total area of the surface of the adult is 1.0 sq.cm., hence at 0.2 atm. oxygen tension the maximum uptake would be, using the mean value for permeability above, 6.1 cu.mm./hr., which is identical with the basal metabolic rate.

It is of interest to compare the values of cuticle permeability to oxygen with those deduced from other investigations. Krogh, using preserved material from *Oryctes* larvae, gives 1.3×10^{-2} c.c./min./1 μ thickness. Fraenkel & Herford (1938), working on the cutaneous respiration of *Calliphora* larvae, give oxygen consumptions for larvae in which the spiracles are blocked by ligaturing. These figures give a diffusion resistance of 6×10^4 . Measurements carried out on larvae of the same species gave the total surface area as 1.0 sq.cm. and the cuticle thickness about 60 μ , from which it is possible to calculate the absolute permeability. Table 4 summarizes these results.

It will be seen that the permeability of the nymphal cuticle is of the same order as the soft cuticle of *Calliphora erythrocephala* larvae, whereas the adult, whose cuticle is harder and more darkly pigmented (a feature usually associated with a greater degree of cross linkage), is of much lower permeability. Krogh's data,

which are taken from isolated cuticle, are of doubtful physiological significance, as the material had been preserved for some time in alcohol.

The lower permeability in the adult would in itself make necessary some auxiliary respiratory mechanism. But there is no obvious advantage to the adult, at least in the form occurring in Britain which is wingless and purely aquatic, in not maintaining a high degree of cutaneous respiration at the same time as evolving a plastron mechanism. On the other hand, we have shown (Part I) the necessity for a rigid structure bounding the animal to take the strain imposed by a lower internal oxygen tension than that existing outside. It is likely that a rigid exocuticle capable of withstanding this is not easily compatible with one of high permeability, and the evolution of the plastron may therefore to some extent involve a reduction in cutaneous respiration. This will help to explain the sudden and complete switch-over after the fifth instar larva.

Table 4

Investigator	Cuticle	Cuticle thickness (μ)	Permeability (c.c./sec./sq.cm.)
Krogh	<i>Oryctes</i> larva	—	0.22×10^{-7}
Fraenkel & Herford	<i>Calliphora</i> larva	60	1.0×10^{-7}
Thorpe & Crisp	<i>Aphelocheirus</i> nymph	45	1.6×10^{-7}
	<i>Aphelocheirus</i> adult	45	0.38×10^{-7}

6. RESPIRATION THROUGH THE PLASTRON

(a) *Diffusion resistance of the plastron determined experimentally*

The diffusion resistance of the adult cuticle can be calculated from its area and permeability. Taking the latter as 0.85×10^{-5} c.c./sec./sq.cm./atm., and the area as 1.0 sq.cm., the diffusion resistance will be about 12×10^4 in c.c./sec./atm. The diffusion resistance of the adult measured experimentally was found to be not greater than 1.9×10^4 units. Since these two routes are in parallel, we may write

$$\frac{1}{R} = \frac{1}{R_{\text{cuticle}}} + \frac{1}{R_{\text{plastron}}},$$

where R is the diffusion resistance of the normal adult. From this relation the plastron resistance is found to be not greater than 2.3×10^4 units.

As a check on this figure, and in order to ascertain where the main diffusion resistance occurs, we have attempted to evaluate the drop in tension at each step in the diffusion path from the external supply in the water to the tissues. Much of the calculation below depends on making reasonable assumptions, but since the over-all figure obtained agrees reasonably well with the experimental data, we consider that the detailed calculations are qualitatively correct and indicate where resistance to diffusion chiefly occurs. The calculation further serves to prove that no physiological process of secrétion, or any process involving expenditure of energy is necessary to enable the plastron to function, but that diffusion offers a sufficient mechanism.

(b) Calculation of diffusion of oxygen through the plastron into the tissues

In calculating the diffusion of oxygen into the tissues of *Aphelocheirus*, via the plastron and tracheal system, it is convenient to divide this path as follows into a number of steps:

- (i) From the external medium into the plastron.
- (ii) From the plastron to the spiracles.
- (iii) From the spiracles to the tracheal endings.
- (iv) From the tracheal endings into the tissues.

At each step the drop in tension when the respiration is at its basal level (6 cu.mm./hr.) is calculated.

(i) Diffusion into the plastron

The diffusion of gases across a gas-water interface was originally supposed to be subject to a definite physical barrier leading to a drop in tension at the interface. Earlier workers such as Krogh and Ege have measured the rate of gas exchange under 'ordinary' conditions and supposed that an invasion coefficient I , specific to each gas, could be defined as $q = \Delta p I$, where q is the quantity of gas passing per second and Δp is the difference in tension between gas and liquid.

This drop in tension across the interface is now considered to be wholly due to inefficient mixing at the surface layers, and, indeed, Krogh (1919) has later shown that the value of I could be increased indefinitely by more vigorous stirring. It is clear that the apparent drop in tension at the interface is due to the existence of thin diffusion shells of the medium through which the gas must penetrate, prior to reaching the layers of liquid in which convection is sufficiently rapid to mask diffusion. Hence it would be more appropriate to employ a universal constant C , related to the convectational conditions at the surface and the permeability of the medium P , thus $I = CP$.

It is well known that a fluid medium flowing past a small stationary object forms a 'boundary layer' at the surface of the object, in which the viscous forces solely operate. This layer may be treated from the diffusion aspect as an approximately stationary layer whose thickness depends on the dimensions of the object, the velocity of flow and the kinematic viscosity of the medium. Using such an approximation $C = 1/d$, where d is the average boundary layer* thickness. Hence $\Delta p = qd/P$. From this relation we have calculated from Ege's figure for the invasion coefficient of oxygen of 0.029 c.c. at N.T.P./cm.²/min./atm. that under his conditions of experiment the boundary layer was about 12×10^{-4} cm. in thickness. His figure for nitrogen is 0.009, giving $d = 20 \times 10^{-4}$ cm., which is not in agreement, possibly owing to slightly different experimental conditions. It is of interest to compare these figures with those of Schulman & Teorell (1938), who measured the diffusion boundary layer thickness at a solid-liquid interface and obtained a figure of 30×10^{-4} cm.

It is likely that Ege's value of I applies to somewhat static conditions, and while

* This will be strictly a diffusion boundary layer, and will not necessarily have identical form with the hydrodynamical boundary layer.

recognizing its defects, we have used it to give an approximate order for the tension drop. With good convection this tension drop would obviously be somewhat less than the value obtained.

When respiration takes place across the plastron two diffusion processes occur at right angles to each other:

- (a) Invasion across the water-air boundary.
- (b) Diffusion across the plastron.

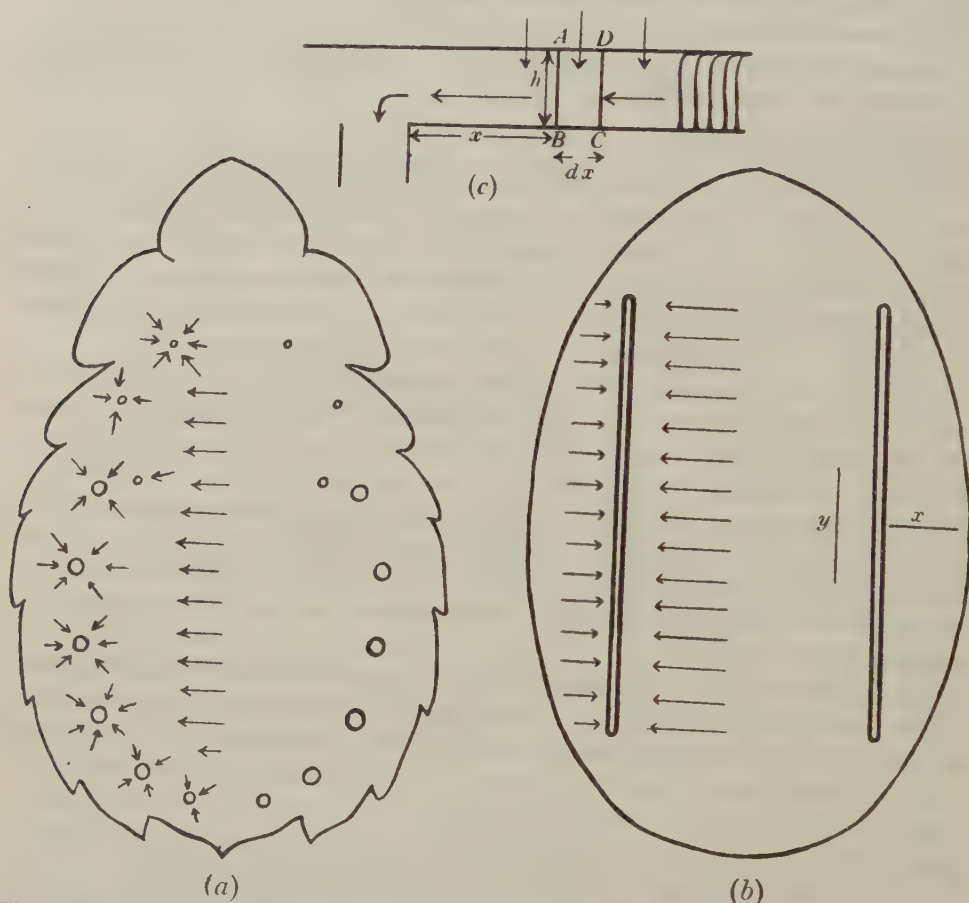


Fig. 3. Diagram of oxygen diffusion across the plastron. (a) Actual paths of diffusion. (b) Idealized system treated in Appendix I. (c) Section of plastron showing invasion across $A-D$ and diffusion along the ' x ' axis.

It is clear that the plastron will not contain oxygen at a uniform tension; there will be a steady fall towards the spiracular orifices. It is important to know how this fall in tension will affect the plastron in its function as a gill, i.e. whether absorption of oxygen takes place all over the plastron or only close to the spiracles.

Analysis of the above-mentioned processes may be made if we treat the line of spiracles down each side of the animal as a groove into which oxygen diffuses in a direction at right angles to the long axis of the body (Fig. 3 *a, b*).

This treatment, given in Appendix I, leads to a relation for Δp , the deficiency of plastron tension from surroundings (p in Appendix) in terms of x the distance from the line of the spiracles

$$\Delta p = A [Be^{nx} + C \sinh nx], \quad (4)$$

where A , B , C and n are constants defined in terms of i_0 the invasion coefficient, D the diffusion constant of oxygen in the hair pile, q the rate of respiration, and h , y , x_1 the physical dimensions of the plastron.

In determining the value of p the values of i_0 and D must be modified to allow for the influence of the hairs. The size of the hairs is comparable with the mean free path of the gas molecules in the plastron (mean free path = 9×10^{-5} cm., radius of hair = 10^{-5} cm.); hence a considerable proportion of the collisions will be between the gas molecules and hairs. At any one plane transverse to the direction of flow of the gas, 0.4 of the available space will be blocked by hairs. Hence D will be about 0.10 rather than 0.18 sq.cm./sec. The surface of the water will be reduced by the presence of the tips of the hairs, but, owing to the bulging of the menisci as shown in Fig. 23 of Part I, this effect is less than might be anticipated. The second effect on i_0 is likely to be due to the reduction in flow of the water and the resistance to diffusion of stagnant regions between the hair tips. The value of i_0 , the invasion coefficient, is equivalent to a static water film of thickness 1.2×10^{-3} cm. The size of the hair tips is only 2.0×10^{-5} cm. diameter, and hence their effect on resistance is negligible in comparison with that of the boundary layer. The effect of the hairs on flow will similarly be negligible. It seems likely, therefore, that a reduction of i_0 to half its value at a plane air-water surface is an outside limit. In the calculation h is taken as 5×10^{-4} cm., $x = 0.25$ cm. and $y = 0.60$, from measurements on *Aphelocheirus*. This would give the assumed effective area of the plastron as 60 sq.mm.

In Fig. 4 the tension Δp is plotted against distance from the spiracular openings. It will be seen that the shape of the curve is not very sensitive to variations in i_0 and D , and that within the likely range of these values the plastron tension is fairly uniform. Naturally there is greater uptake close to the spiracle, but the error in considering the plastron as being at a uniform tension (shown by broken lines in Fig. 4) is very small. Clearly the plastron is efficient over the whole surface of the bug.

Giving i_0 a probable value of 4×10^{-4} , the mean value of Δp over the plastron will be 0.7% atm., below its surroundings when $q = 1.67 = 10^{-6}$ c.c./sec., while the pressure drop along the plastron, when $D = 0.10$ c.g.s. unit, is about 0.1% atm. (Fig. 4) giving a total drop of 0.8% atm. to the line of the spiracles.

(ii) Diffusion from the plastron to the spiracles

It will be seen from subsequent data that the spiracles do not necessarily absorb equal supplies of oxygen; it follows that local oxygen tensions round each spiracle will vary. The simplest method of approach is to treat the plastron area close to the spiracular rosette as a diffusion resistance in series with the main tracheal branch leading from the rosette. To calculate this resistance from the outer edge to the centre of the rosette the relation

$$R = [1/2\pi Dh] \times \log_e \frac{r_1}{r_0}$$

may be employed. r_1/r_0 has an average value of about 40 giving $R = 12 \times 10^3$ units. This value will be employed below.

(iii) *Diffusion through the tracheal system*

The complete analysis of the tracheal system would involve infinite labour, but fortunately a greatly simplifying relation, first shown by Krogh (1920) to be true of *Tenebrio*, is also true of *Aphelocheirus*. The tables below, based on careful analysis of trunks supplying muscle and gut, show that the total cross-sectional area of a

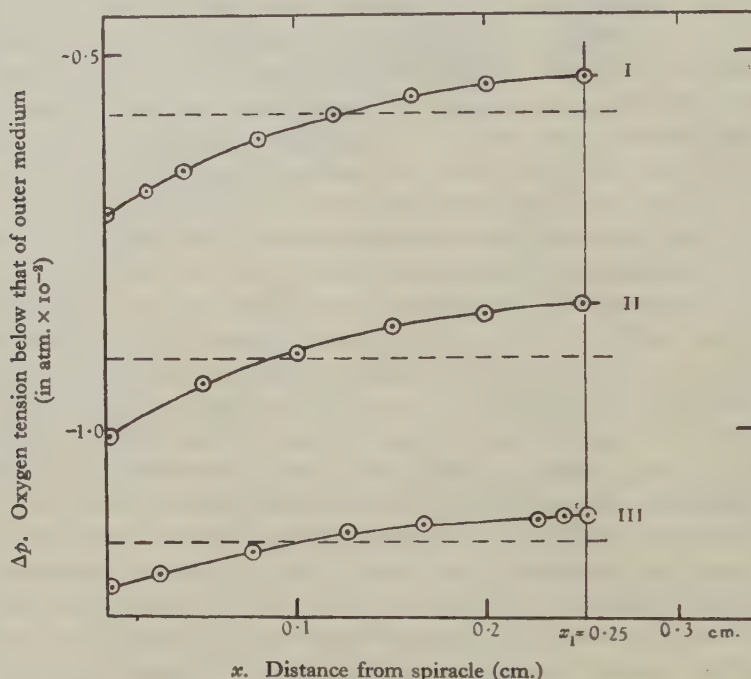


Fig. 4. Variation of oxygen tension in the plastron of *Aphelocheirus* at different distances from the spiracles (broken lines indicate tension if plastron assumed to be uniform [$D=0$]):

$$\Delta p = A [Be^{nx} + C \sinh nx].$$

Oxygen uptake assumed to be 6.0×10^{-3} c.c./hr. I. Diffusion constant D in plastron $= 0.10$ sq.cm./sec.; invasion coefficient $i_0 = 4.8 \times 10^{-4}$ c.c. at N.T.P./cm.²/sec./atm./cm. II. $D = 0.10$, $i_0 = 3.0 \times 10^{-4}$. III. $D = 0.18$, $i_0 = 2.4 \times 10^{-4}$.

trunk remains unaltered when it is subdivided into finer ramifications. All trunks were assumed to be circular in cross-section, and their diameters were measured by means of a micrometer scale eyepiece. This relation may be expressed

$$\pi R^2 = \sum_S \pi r_S^2,$$

where R is the radius of the main trunk and r_S is the radius of a subsidiary trunk. This relation makes possible the estimation of the diffusion resistance of a tracheal ending from a measurement of the diameter of the main trunk and the distance l , from the origin to the mean termination of the tracheoles.

Two further assumptions were made:

(1) That absorption of oxygen occurred entirely at the tracheal endings. This assumption will give a maximum drop in tension, as it ignores any uptake of oxygen via the main tracheal walls and body fluid.

(2) That the absorption of oxygen by tissues was proportional to their tracheal supply. Though not true at any instant when one particular set of organs are in use,

Table 5 (a). *Analysis of vessel to head musculature*

Distance (x) along trunk (cm.)	Diameter of subsidiary trunks (D) cm.	$\Sigma \pi r^2$ Total cross-sectional area cm. ²
0.106	0.0098	7.5×10^{-5}
0.117	0.0102	8.15×10^{-5}
0.1275	0.0068	9.2×10^{-5}
	0.0017	
	0.0017	
	0.0081	
0.138	0.0068	7.2×10^{-5}
	0.0017	
	0.0017	
	0.0047	
	0.0038	
	0.0013	
	0.0010	
0.1445	0.0068	7.3×10^{-5}
	0.0017	
	0.0034	
	0.0030	
	0.0017	
	0.0038	
	0.0013	
	0.0010	

Table 5 (b). *Analysis of vessel from first thoracic spiracle*

Distance (x) along trunk (cm.)	No. of subdivisions	$\Sigma \pi r^2$ (cm. ²)
0	1	2.4×10^{-5}
0.0064	2	2.1×10^{-5}
0.038	2	2.7×10^{-5}
0.040	3	1.7×10^{-5}
0.049	6	2.8×10^{-5}

Table 5 (c). *Analysis of vessel to oesophagus and crop from third thoracic spiracular vessel*

Distance (x) along trunk (cm.)	No. of subdivisions	$\Sigma \pi r^2$ (cm. ²)
0	1	0.205×10^{-5}
0.017	3	0.182×10^{-5}
0.036	7	0.166×10^{-5}
0.093	16	0.136×10^{-5}

it is reasonable to suppose that the tracheae are developed in accordance with the average oxygen economy of the tissues they supply.

In calculating the probable passage of oxygen under diffusion gradients in the tracheal system, Kirchhoff's laws, applicable to networks of electrical resistances, may be applied to the tracheal tubes treated as diffusion resistances. The diffusion resistance of the main tracheal trunks and the resistance of the branches terminating in the tissues are calculated from the relation

$$R = \frac{1}{D} \frac{l}{\sum_s \pi r_s^2},$$

where D is the diffusion coefficient of oxygen in air. The result of this calculation is shown in Figs. 5 and 6. Fig. 5 gives the resistances and tensions through the main divisions of the tracheal system, while Fig. 6 shows the distribution of tensions at the tracheal endings. It will be noted that the tension drop in the tracheal system itself is very small, the mean value being of the order of 0.3% atm.

(iv) *Diffusion into the tissues from tracheal endings*

The drop in tension from the tracheal endings into the tissues is not readily calculated owing to the large number of unknown factors. We have studied the tracheal endings and their distribution in muscle and gut in order to determine this pressure drop, but the values obtained cannot be considered to indicate more than an approximate order of magnitude. To treat the problem one of us (D. J. C.) has developed a number of equations for diffusion of oxygen in a medium which is absorbing it simultaneously (see Appendix II).

Four representative tissues were studied on the assumption that these were typical for the whole animal. These were (1) hindgut, (2) crop, (3) dorsoventral thoracic muscle, (4) leg muscle (third thoracic).

The value of the absorption of oxygen (m) was computed to be in the order of $1.0-1.5 \times 10^{-4}$ c.c. O_2 /sec./c.c. of living tissue; the permeability of the tissues P is taken from Krogh's (1919) data as 2.3×10^{-7} c.c./sec./cm.²/atm./cm.

Table 6

Tissue	Mean dimensions				Value of m taken (c.c./sec./c.c. of tissue)	Equation employed (see Appendix II)	Tension drop from tracheole (atm. %)
	r_0 (μ)	r_1 (μ)	t	R (μ)			
Hindgut	0.15	3.4	35	—	1×10^{-4}	(17)	3.1-3.3
Crop	0.15	10-12.5	25	—	1×10^{-4}	(17)	3.1-3.6
Thoracic muscle	0.15	10	—	25	1.5×10^{-4}	(10) and (18)	1.2-2.8
Leg muscle	0.15	8	—	35	1.5×10^{-4}	(10) and (18)	0.7-4.9

The results of these calculations are shown in Table 6. The table indicates that the maximum drop in tension from the tracheae and to the tissues is in the order of 3%. This figure represents the tension drop at points most remote from the tracheoles, so that the average value will be rather less than this. Also it should be remembered that some diffusion occurs through the walls of the larger tracheae and via the body fluids which are kept in motion by the heart. This may somewhat

The drop in tension at each step may now be summarized in Table 7. From this over-all drop in tension, the diffusion resistance is found to be about 2×10^4 . This is in sufficient agreement with the observed value (2.3×10^4) to justify the broad

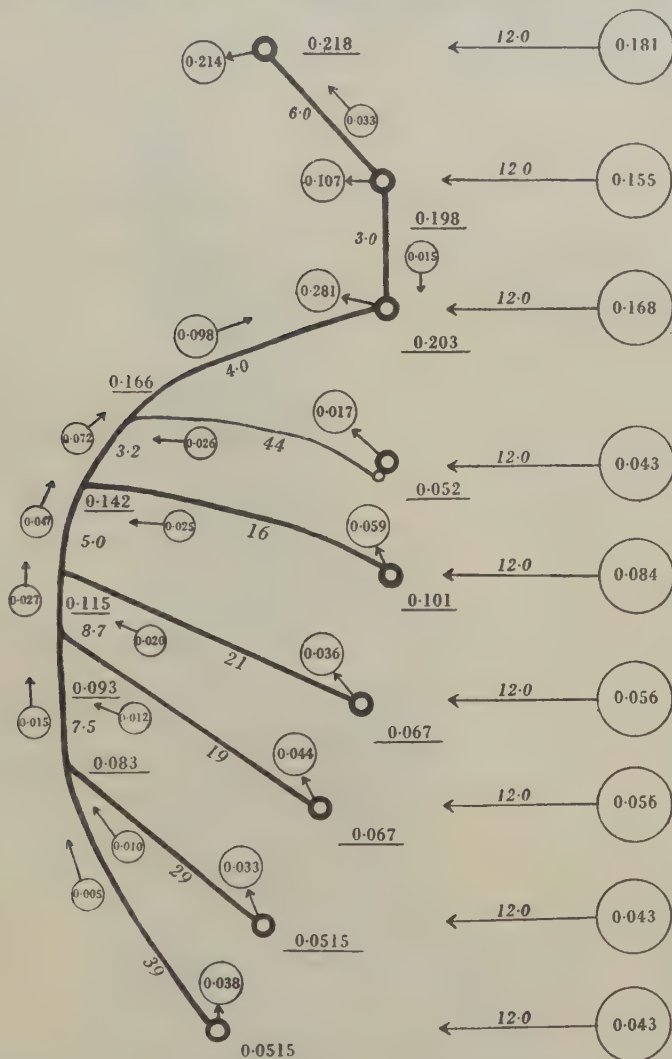


Fig. 5. Diagrammatic representation of spiracles and main tracheal trunks showing the diffusion of oxygen in the tracheal system, calculated according to the assumptions made in the text. Heavy circles represent spiracles. Heavy lines represent main tracheal trunks. (1) Figures in circles indicate amount of oxygen passing through each spiracle (large circles) and tracheal trunk (small circles) in c.c. per sec. $\times 10^{-6}$. (2) Underlined figures indicate fall in oxygen tension below that of the plastron in percentage atmosphere. (3) Figures in italics indicate diffusion resistance into spiracles and through the main tracheal trunks (c.g.s. units $\times 10^{-3}$). The resistance of each rosette is taken as 12.0×10^{-3} c.g.s. units, and the amount of oxygen distributed locally by the small tracheal trunks associated with each spiracle is indicated by an arrow directed away from the spiracle.

Table 7

Diffusion	Δp (atm. %)
Outer medium into plastron	0.7
Across plastron	0.1
Through spiracles and tracheae to tracheoles	0.3
Into the tissues from tracheoles	2.0
Total	3.1

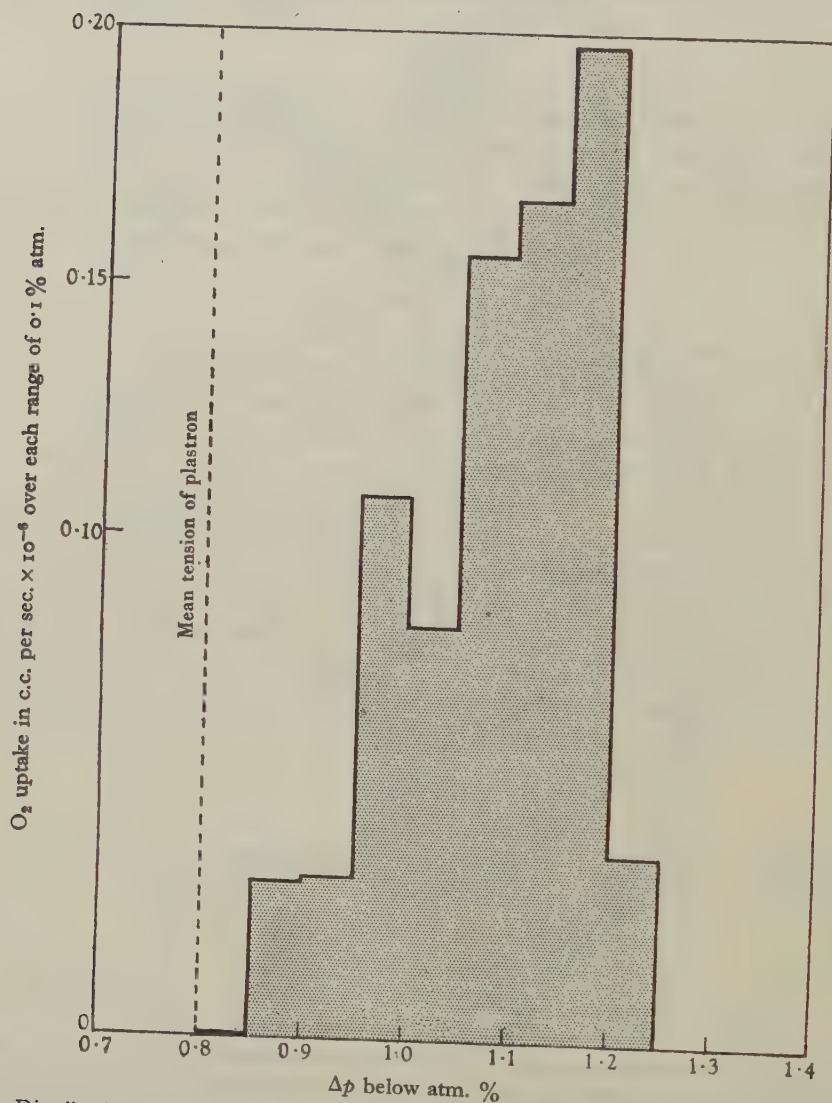


Fig. 6. Distribution diagram illustrating the uptake of oxygen by groups of tracheal endings at various tensions, Δp below that of the surroundings. The shaded area represents the total uptake of oxygen by a single resting animal under normal conditions. Δp is the difference in tension between the tracheal endings and the outside medium expressed in percentage of an atmosphere. The tension at the tracheal endings is seen to lie between 0.85 and 1.25 % atm. below that of the surroundings.

lines of the calculation and to reject any theory which involves selective absorption. It is of interest to note that the tracheal and plastron resistances are very small compared with the resistance on entering the plastron and the resistance from the tracheoles into the tissues.

7. COMPARISON OF RESPIRATION OF ADULT AND NYMPH

A comparison of the data for the adult and the nymph given in Table 8 makes clear the necessity for a supplementary mode of respiration after the fifth instar. The main factors are:

- (a) Decrease in permeability of the adult cuticle (see p. 279 above).
- (b) Increase in activity in the adult.
- (c) Increase in uptake/surface area in the adult.

Table 8. *Comparison of respiration of nymph and adult*

	Fifth instar nymph	Adult
Average weight (g.)	0.025	0.04
Basic oxygen requirement (cu.mm./hr.)	3	6
Oxygen requirement when active (cu.mm./hr.)	10	40
Total surface area (sq.mm.)	75	100
Uptake per sq.cm. (cu.mm./hr.)	4	6
Thickness of cuticle (μ)	45	45
Permeability of cuticle (c.c./sec./sq.cm./atm.)	3.6×10^{-5}	0.85×10^{-5}
Diffusion resistance of cuticle (c.g.s. units)	3.7×10^4	12×10^4
Diffusion resistance of plastron (c.g.s. units)	—	2.3×10^4
Over-all resistance of normal animals (c.g.s. units)	3.7×10^4	1.9×10^4
Respiratory index for cutaneous respiration only (atm. ⁻¹)	32	5
Respiratory index for normal animal (atm. ⁻¹)	32	33
Intact surface required for basal respiration in air-saturated water:		
(a) Area (sq.mm.)	11.5	100
(b) Proportion of whole (%)	15	100
Minimum oxygen tension to maintain basal respiration through cuticle only (% atm.)	3.1	20
Minimum oxygen tension to maintain vigorous movement respiration via cuticle only (% atm.)	10.3	130
Minimum oxygen tension required by normal individual for:		
(a) Basal respiration (% atm.)	3.1	3.0
(b) Vigorous movement (% atm.)	10.3	20
Proportion of respiration by cuticle (% atm.)	100	16

It is also of interest to compare this table with the results of behaviour experiments in which nymphs, normal adults and adults treated with wetting agents, were exposed to water at low oxygen tension (Part I). The oxygen tolerance for adults and nymphs is almost identical. Down to a tension of 3-4% both show an initial shock response to severe oxygen lack, but after about 1 hr. become acclimatized and regain some activity though still not normal, normal behaviour requiring about 5-8% saturation. At 2% saturation both fail to regain activity unless replaced in higher saturation and would eventually die. This result agrees well with the observed critical oxygen concentration of 3% saturation for basal metabolism.

Adults in which the plastron is removed do not appear normal unless kept in air-saturated water, and even there tend to be 'lethargic'. On the other hand, their response to low oxygen tension is not as severe as would be expected. At 8%

saturation they behave subnormally, while at tensions from 5–3% they show progressive deterioration in behaviour but recover slowly in air-saturated water. Their behaviour is thus markedly different from the normal adult, but as the spiracles themselves are not fully closed and some degree of oxygen debt can undoubtedly be tolerated, these experiments do not emphasize the importance of the plastron as clearly as measurements on oxygen uptake. It is also important to note that any

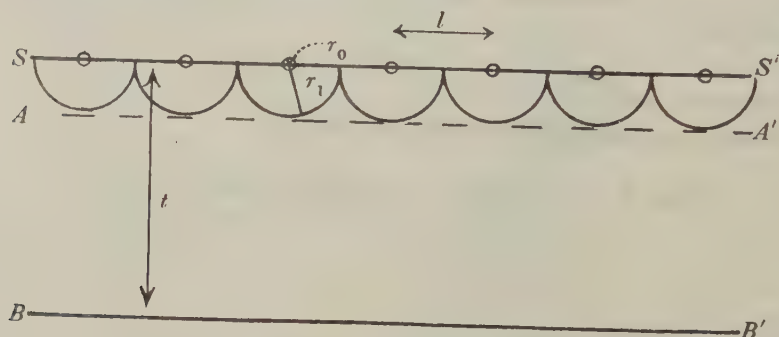


Fig. 7. For explanation see text of Appendix II.

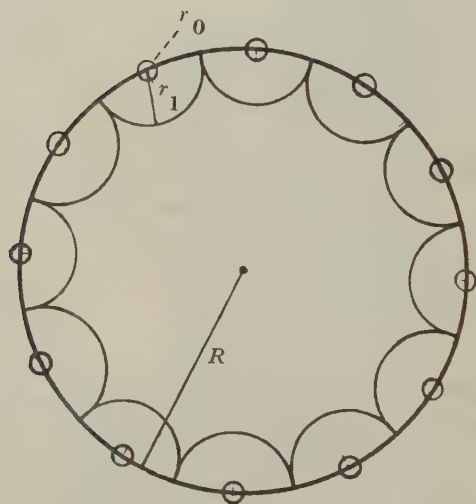


Fig. 8. For explanation see text of Appendix II.

respiratory structure based on diffusion is reversible, and a highly efficient plastron will rapidly reduce the tissues to a low oxygen tension, if this exists in the surrounding medium. Hence the closure of the plastron may well desensitize the animal to conditions of oxygen want, so that its effect is not as pronounced as would be expected in a short interval of time (see calculation in Part III, p. 319).

8. DEVELOPMENT OF PLASTRON IN FIFTH INSTAR NYMPH

In all the nymphal stages the plastron is lacking and the spiracular system is closed—as can easily be proved by immersion of the whole animal in a stained oil under reduced pressure. The cuticle of the earlier nymphal instars is, of course, very much thinner than that of the adult; but as the moults pass it thickens rapidly and by the time the fifth instar is fully developed is in most places nearly equal to that of the adult. The structure of the fifth instar nymphal cuticle differs in many respects from that of the adult, as will be seen from Figs. 9 and 10 and from Pl. 6*d* in Part I.

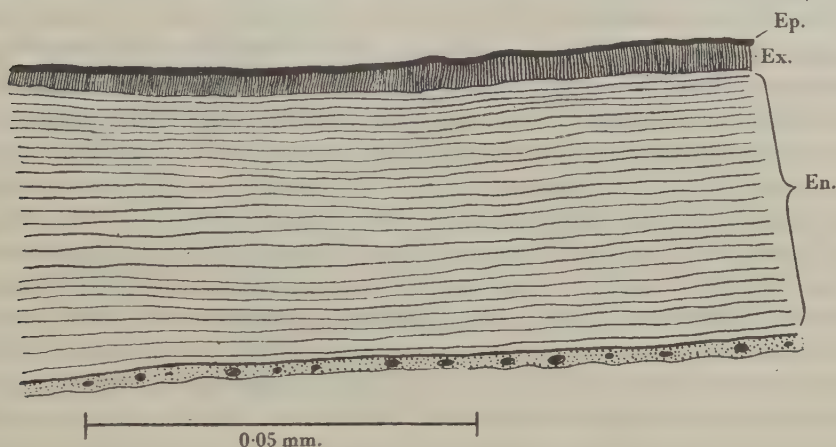


Fig. 9. Cuticle of fifth instar nymph.

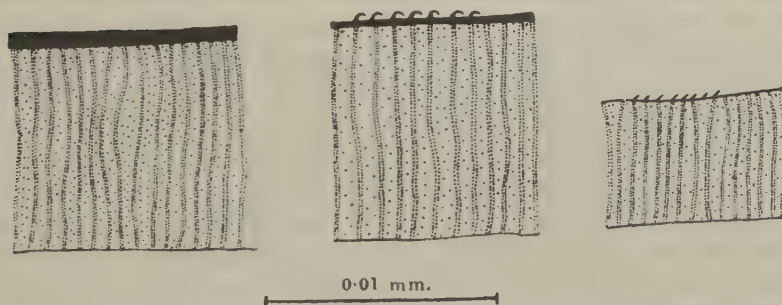


Fig. 10. Variation in structure and proportion of epicuticle of fifth instar nymph.

In the nymph the hypodermis is overlaid by a transparent endocuticle which shows itself in unstained frozen sections as composed of about twenty fairly well-defined horizontal lamellae. This in its turn is covered by an exocuticle of a moderate degree of pigmentation in which pore canals or something similar to them can be clearly seen. Overlying this again comes a relatively thick epicuticle which in many places and with all but the highest magnifications appears structureless. Under good conditions, however, some sections show in places an appearance as if the pore canals of the exocuticle are continued through the epicuticle, giving rise on its

surface to exceedingly minute spines, sometimes curved and sometimes straight (see Fig. 10). These are the only structures in the nymph which in any way resemble the plastron hairs on the adult. The epicuticle varies a good deal in thickness from place to place and one frequently finds spots where it can hardly be seen at all, in which case the exocuticle with its pore canals looks like a very stiff hair pile not containing air but fully wetted. This, however, appears to be an artefact. Although the exocuticle often shows brilliantly in section with a dark background illumination, this is not due to any contained air, since neither strong butyl alcohol nor cetyl pyridinium chloride will alter the appearance of either sections or the whole insect, even after an hour's treatment. At any time during the period of digestion and reabsorption of the nymphal endocuticle the layers of adult endocuticle can be seen in process of formation beneath it. The exocuticle first appears as a structureless layer in which close-packed transverse laminae are soon seen. These become more conspicuous externally, this process seeming to constitute the early differentiation of the epicuticle (cf. Wigglesworth, 1933). The separation of these laminae by splitting gives rise to the hair-pile system of the future plastron, but the hairs are not at this stage obviously bent over at the tips. This may, however, in part be due to difficulties in microscopic observation of such minute and delicate structures. The fact that the hair pile of the adult is well formed before much of the old endocuticle is absorbed, seems to show quite clearly that the waterproofing of the plastron is a relatively late stage in adult cuticle formation. When all but about one layer of the old endocuticle has been reabsorbed, the moulting fluid decreases in quantity and a pale sheen appears first on the ventral part of the meso- and metathorax and in the region of the intersegmental membrane of the abdomen, suggesting that with the commencement of the process of absorption of the moulting fluid a gas is coming from solution and invading the plastron. There is no suggestion at this early stage of any continuity between the gas layer developing in the plastron and the gaseous contents of the tracheal system which still appears closed. The sheen begins to show through the transparent nymphal cuticle at a time varying from 48 hr. to a few days before the moult is actually due. When the insect is nearing moult the sheen spreads more rapidly and is particularly conspicuous in the region of the rosettes and of the organs of pressure sense where it may show brilliantly under the old skin. Nymphs do not come to the surface to moult but in the aquarium cling to plants or stones some distance below the surface. The skin breaks by means of two splits at right angles forming an inverted $\perp$ at about the level of the second abdominal segment. Cast skins showing tracheal linings are found attached to the points on the abdomen beneath each rosette and also to the sites of the thoracic spiracles, and the adult comes out into the water with the plastron surface perfectly dry and complete. That not even contact with gas bubbles in the water is necessary to start the process of aeration of the plastron is shown by the fact that we have had perfectly good specimens with sheen complete produced during experiments in which access to the surface or to gas bubbles was prevented.

The process of waterproofing of the hair pile raises an interesting physico-chemical problem. Although qualitatively this phenomenon is not different from

many examples of the filling of closed tracheal systems with gas, the surface forces involved are very much greater owing to the large surface area of the hairs and the small size of the gas space between them (see Part I). Normally at a solid-water interface, any mobile polar groups become orientated towards the water and non-polar groups away from it, since in this manner the free energy of the system is minimal. If the surface is dried reorientation may occur and the non-polar groups become external and the surface consequently less wettable. It might be suggested that gas secretion into the plastron would expel the moulting fluid from the surface and thus render it hydrophobic by the above mechanism. This view seems untenable since:

(i) Secretion of gas would have to take place against an excess pressure of at least 4 atm. (Part I). This does not seem likely.

(ii) Even if gas were secreted the surface is still saturated in aqueous vapour and an adsorbed layer of water would be present, hence waterproofing would not occur.

It is necessary therefore to assume that some structural modification of the external molecular layer of the epicuticle occurs whereby hydrocarbon groups are directed outwards even though water is present at the interface. The production of an unwettable layer would automatically drive out the water from the plastron, since the surface forces would then be reversed. Two possibilities might be conceived. Either this hydrophobe layer is formed during the development of the epicuticular hairs and a secretion of gas into the moulting fluid induces the appearance of gas; or alternatively, a chemical change in the epicuticular surface, making it hydrophobic, causes the moulting fluid to retreat from the plastron.

9. THEORY OF PLASTRON RESPIRATION

The maintenance of a difference in pressure between the plastron space and the surrounding medium based on the mechanical principles outlined in Part I, gives the plastron a functional resemblance closer to the closed tracheal gill than to the air store, though the latter may, to a limited extent, behave as a physical gill.

For this reason, the invasion of nitrogen does not play the important role that Ege (1918) has emphasized in connexion with the air store; but the necessary conditions for the tracheal gill that the walls of the tracheal air sacs, etc., should be capable of withstanding such pressure as the diffusion gradient may impose, as shown by Koch (1936), are equally relevant to the plastron.

We may develop the ideas of these authors more rigidly as follows:

Let us assume with Krogh (1910) that the passage of a gas g across a liquid-gas interface demands an 'invasion coefficient' i_g , so that the amount passing

$$\frac{dg}{dt} = Ai_g(t_1 - t_2),$$

where A = area of the surface, and t_1 and t_2 the tensions of the gas on either side.

The carbon dioxide tension is everywhere maintained at a very low level on account of its solubility in the surrounding medium and the buffering action of the

body fluids. The water-vapour tension is almost constant throughout the system and may also be neglected in the treatment outlined below.

Let us first consider an air store of volume V , area of surface A . Let t_0 and t_n be the tension of oxygen and nitrogen in the outside medium and p_0 and p_n the partial pressures in the air store. The invasion coefficients of oxygen and nitrogen are i_0 and i_n , and the metabolic consumption of oxygen q . The net gain of oxygen by the air store will be

$$\frac{dO}{dt} = Ai_0(t_0 - p_0) - q \quad \text{c.c./sec.} \quad (19)$$

The net gain of nitrogen similarly will be

$$\frac{dN}{dt} = Ai_n(t_n - p_n). \quad (20)$$

We shall now assume that the pressure exerted on the walls of the air store is constant, equal to P , and equals the sum of the gas tensions inside, since the air store, by definition, cannot withstand a pressure difference on its walls. Thus

$$p_0 + p_n = P. \quad (21)$$

Combining (19), (20) and (21),

$$\frac{dv}{dt} = \frac{dO}{dt} + \frac{dN}{dt} = A(t_0 i_0 - i_n(P - t_n) - p_0(i_0 - i_n)) - q. \quad (22)$$

From equation (22) it can be seen that the greater the pressure P on the system, the more rapid the decrease in volume. This equation will be employed in a later publication in which the behaviour of air stores in aquatic insects will be considered.

Equations (19) and (20) may now be used for conditions in the plastron. If the gas exchange has reached a steady state, since the volume of the plastron does not alter,

$$\frac{dO}{dt} = \frac{dN}{dt} = 0. \quad (23)$$

Hence

$$t_n = p_n, \quad (24)$$

and

$$p_0 = t_0 - \frac{q}{Ai_0}. \quad (25)$$

The total pressure within the plastron p is therefore

$$p = p_0 + p_n = t_0 + t_n - \frac{q}{Ai_0}. \quad (26)$$

If the external pressure on the outer surface is P , and the depth of immersion of the animal is h , ρ being the density of water and g the acceleration of gravity, the difference of pressure which must be maintained by the plastron Δp will be

$$\left. \begin{aligned} \Delta p &= P + h\rho g - p, \\ \Delta p &= P + h\rho g - t_0 - t_n + \frac{q}{Ai_0}. \end{aligned} \right\} \quad (27)$$

This formula is an elaboration of that given by Krogh (1910) and Ege (1918).

Normally under saturated conditions, $P = t_0 + t_n$; but a tension difference is still necessary to maintain respiration on account of the pressure and solubility increase with depth ($h\rho g$) and the diffusion resistance q/Ai_0 .

The very small effect of increase in pressure on the chemical potential of dissolved oxygen has been ignored, since it would not affect these conclusions at depths normally considered.

In order to show the magnitude of the pressure difference that must be sustained, Table 9 has been inserted. The calculations apply to the bug *Aphelocheirus*, in which the plastron has an area of 100 sq.mm., and a basal metabolic rate of 6 cu.mm./hr. The 'active' metabolic rate is assumed to be 10 times this value.

The efficiency of the plastron in maintaining an effectively closed tracheal system and preventing the invasion of water must be clearly distinguished from its efficiency as a respiratory organ. The maintenance of the pressure difference Δp for a respiration rate q is a necessary condition for respiration, but it is also necessary that the

Table 9

Condition	Tension of nitrogen t_n in outer medium (atm.)	Tension of oxygen t_0 in outer medium (atm.)	Depth h (m.)	External pressure of atmosphere P (atm.)	Internal oxygen tension p_0 (atm.) for basal metabolism	Δp required for basal metabolism (atm.)	Internal oxygen tension p_0' (atm.) for great activity	$\Delta p'$ required for great activity (atm.)
Unsaturation in oxygen	0.8	0.2	0	1.0	0.1965	0.0035	0.165	0.035
	0.8	0.1	0	1.0	0.0965	0.1035	0.065	0.135
	0.8	0.05	0	1.0	0.0465	0.1535	0.015	0.185
	0.8	0.0	0	1.0	-0.0035*	0.2035*	-0.035*	0.235*
Supersaturation in oxygen	1.0	0.25	0	1.25	0.2465	0.0035	0.215	0.035
	1.0	0.25	0	1.0	0.2465	(-0.2465)	0.215	(-0.215)
Effect of depth	0.8	0.2	2	1.0	0.1965	0.1985	0.165	0.230
	0.8	0.2	10	1.0	0.1965	0.98	0.165	1.01
	0.8	0.2	50	1.0	0.1965	4.88	0.165	4.91
Unsaturation in nitrogen and oxygen	0	0.2	0	1.0	0.1965	0.8035	0.165	0.835
	0	0.10	0	1.0	0.0965	0.9035	0.065	0.935
	0	0	0	1.0	-0.0035*	1.0035*	-0.035*	1.035*

oxygen diffusion gradient through the animal is large enough to maintain this rate q . In equations (23)–(27) we have been concerned solely with pressure equilibrium and we have assumed that this was so. Calculation of p_0 , the tension of oxygen in the plastron, shows that when the oxygen tension t_0 outside the animal is low, p_0 may require to be negative to maintain respiration. In practice, of course, p_0 must have a positive value depending on the internal diffusion resistance, so that the conditions marked with an asterisk (*) in Table 9 are hypothetical, oxygen want being the limiting factor to the animal's existence.

It should also be noted that the above treatment assumes a uniform tension of oxygen throughout the air store or plastron. This has been shown to be approximated to by *Aphelocheirus* (see § 6 (b) (i)), and in a larger air store the lack of uniformity would be quite insignificant.

Several interesting and significant points can immediately be seen from this table. Unsaturation of the surrounding medium whether in oxygen or nitrogen has the same effect in increasing the pressure which the plastron must withstand if it is still to function as a gill. This effect of unsaturation is most marked, of course,

when the surrounding fluid is gas-free; the plastron must then withstand the full pressure of the atmosphere and the hydrostatic head, minus, of course, the small pressure of aqueous vapour which has been omitted from the treatment for simplicity.

Should the insect descend to great depths, the strain on the system will be considerable, a limitation already pointed out by Koch (1936) in the case of a closed tracheal system, of which the plastron is really a special case. In this connexion it should be explained that not merely the plastron, but the whole of the tracheal system including air (i.e. tracheae, tracheoles, air sacs and tracheal endings), must be capable of withstanding the excess pressure Δp . In practice, of course, some of this pressure may be taken up by the elasticity of the external cuticle, some by the elasticity of the tracheal walls themselves; but a single defect allowing the volume of the air space to decrease continuously will render the whole system ineffective. However, a small change in volume allowing cuticular elements and tracheal endings to take up the strain would be followed by a limited solution of oxygen and nitrogen until equilibrium gas exchange was re-established.

10. THE EFFICIENCY OF THE PLASTRON IN RELATION TO ENVIRONMENTAL CONDITIONS

The above discussion gives a fairly complete picture of the manner in which the plastron operates. We must now consider how changes in the environment may upset the efficient working of the organ and what degree of latitude is allowed.

The main dangers may be divided into two classes:

- (a) Breakdown in the waterproofing structure with consequent asphyxia.
- (b) Breakdown in respiration in spite of plastron remaining uninjured.

(a) The plastron in *Aphelocheirus* is wetted by surface-active materials which reduce the contact angle of the water-hydrocarbon surface to about 60° , corresponding to a surface tension of about 25 dynes/cm. This is much lower than any value likely to be met in nature. Since the habitat is normally a swiftly flowing stream, where the surface tension will be close to its maximum value, this danger is quite negligible. Even in static water a surface tension of less than 40 dynes/cm. is very unlikely. The only substance producing a sufficient lowering of contact angle would be relatively strong solutions of organic alcohols, acids, etc., or soaps; none of these occur in natural waters.

The second manner in which wetting might occur is on account of increase in depth, causing a rise in the pressure Δp , which must be sustained by the plastron. The breakdown pressure is, however, in the order of 4 atm., corresponding to a depth of 120 ft., which is quite outside any likely habitat. Even if the water is impure ($\gamma = 40$ dynes/cm., $\theta = 90^\circ$) a pressure of 1 atm. is allowed corresponding to 30 ft. in depth. These conditions would only occur in deep ponds or lakes, and it is doubtful if stagnant water with this amount of impurity would contain sufficient oxygen for *Aphelocheirus* to survive.

Another danger affecting insects which descend into deep pools or lakes is the effect of hydrostatic pressure on the tracheole endings. Normally the hydrostatic pressure is balanced by osmotic pressure of the tissues (Wigglesworth, 1930).

Increase in external pressure would force minisci farther away from the tracheole endings, and this would presumably lower the respiratory efficiency. Though we have no evidence on this specific point it has been demonstrated experimentally that the living animal can withstand sudden excess pressure of $4\frac{1}{2}$ atm. for at least 2 min. Although the adults exhibited an attitude of asphyxia when the pressure was increased to $2\frac{1}{2}$ atm., nymphs were still active at the higher pressure, and both recovered rapidly and behaved entirely normally after a few hours, no permanent harm having been done. The behaviour of the adults under these very artificial conditions suggests a shock effect. Hence the animals can withstand considerable depths, but they would be clearly put at a disadvantage if suddenly swept to a depth of 50–60 ft.

The effect of lack of oxygen in the surrounding medium will not greatly affect the breakdown pressure (see Table 9), since the maximum increase in Δp (in oxygen-free water) is only 0.2 atm., provided nitrogen is present in saturation. It is very unlikely that nitrogen would be deficient.

Old individuals are sometimes found in which continual abrasion of the belly has worn off the hair pile leaving dark patches on the mid-ventral line. Such areas will not be employed to collect oxygen, but since the whole dorsal and ventral plastron surface has been shown to be efficient as a physical gill, the loss in efficiency will be proportional to the area of these patches which is usually small compared with the whole plastron surface.

(b) The second danger is one which would be encountered under conditions of low oxygen tension. Two independent types of experiment give about 3% saturation as a lower limit for survival of nymphs, while for normal activity the figure is probably closer to 10–15% saturation. It is unlikely that the oxygen content of rapidly flowing streams would often fall as low as 10%. Butcher, Pentelow & Woodley (1937, p. 1427) have demonstrated that in the Rivers Lark and Itchen the oxygen concentration falls regularly at night, though rarely as low as 8% saturation (0.08 atm. tension oxygen), except perhaps in very sluggish regions or deep holes. On the other hand, conditions in lakes are less favourable. Birge & Juday's figures for dissolved oxygen in Lake Mendota (1911) show that below 10 m. the tension was less than 3% atm. (<1 c.c./l.). Present records of *Aphelocheirus* from lakes do not suggest that it wanders far from the shore. In ponds and still waters with much vegetation the situation would of course be very different, for Butcher *et al.* (1930) have shown that in addition to the effect of decaying organic matter in the mud, living plants can at night very rapidly depress the oxygen content. This factor alone seems fully sufficient to account for the absence of *Aphelocheirus* from such an environment.

The plastron of the adult therefore appears to be a satisfactory organ for its normal environment, and the dangers to which it is exposed are not greater than those which obtain for insects carrying tracheal gills. Since the plastron requires one diffusion gradient less than the tracheal gill (through the outer cuticle) it is relatively more efficient, provided a safe mechanism has been evolved, as in *Aphelocheirus*, to avoid waterlogging.

But while the plastron system of the adult *Aphelocheirus* is thus comparable in efficiency and adaptability to the tracheal gill systems of many aquatic larvae it is really with the respiratory adaptations of other adult aquatic insects that we must compare it if we are to get a true measure of its importance in the life and evolution of the group. When we make this comparison we see at once that the nature of the plastron system is, indeed, a major factor in the ecology of the animal. As a result of the development of this method of respiration *Aphelocheirus* has become independent of visits to the surface. This in turn has enabled it to inhabit swift-flowing streams without danger of being swept away during visits to the surface and, since it does not have to carry an air store, also to become a true bottom-living predator heavier than water. It can thus occupy an ecological niche not available to any other predatory adult insect. On the other hand, of course, its method of respiration necessarily restricts it to well-aerated, that is to turbulent, water.

II. SUMMARY

1. In Part I the ability of the plastron mechanism of *Aphelocheirus* to retain its gas film against a pressure deficiency of several atmospheres was demonstrated, and preliminary experiments were carried out in order to establish that the plastron is in fact respiratory in function. In the present paper quantitative evidence is submitted in support of this view. A comparison is made between the efficiency of the cutaneous respiration of the fifth instar nymph and that of the plastron of the adult.

2. The basic respiratory rate of nymphs and adults was determined by a metal micro-Barcroft respirometer (described in a separate paper, Crisp & Thorpe, 1947) and also by a micro-Winkler method. The basal metabolic rate when the insect is at rest is, for the adult, about 6 cu.mm./hr. O_2 uptake and for the nymph about 3 cu.mm./hr. at 20° C. This corresponds in each case to 150 c.c./hr./kg. live weight. Insects when active in the apparatus increase their respiratory rate six- or sevenfold, and in a strongly swimming adult the increase is probably much more than this.

3. In order to obtain a measure of respiratory efficiency which is independent of the momentary rate of metabolism the concept of 'diffusion resistance' R is employed, where Δp = fall in tension in atmospheres and q = the quantity flowing in units/sec. $R = \Delta p/q$. Another concept, the 'respiratory index', is defined as the reciprocal of the minimum oxygen tension difference between the animal and its surroundings required to maintain the basal respiratory rate. These quantities are determined approximately for *Aphelocheirus* nymphs and adults by measurement of oxygen uptake at low tensions of oxygen in nitrogen. The principles of this method are described.

4. Similar measurements on adults whose spiracles were blocked with low melting-point wax made possible a comparison between the permeability of the cuticle of adult and nymph. The former was found to be 0.38×10^{-7} c.c. O_2 /sec./cm. thickness/sq.cm./atm. difference in tension, and the latter was considerably higher, being 1.6×10^{-7} unit. These values are compared with others found in the literature and are of the same order.

5. The passage of oxygen through the plastron and tracheae of the adult is considered in a number of steps, and the fall in tension calculated at each step. It is shown that the total fall in tension for basal metabolism is about 0.03 atm. on a basis of simple diffusion, making certain necessary simplifying assumptions. This figure agrees with that computed experimentally, at least in order of magnitude, and substantiates the view that the plastron is an efficient respiratory organ over the whole of its surface and operates as a physical gill according to the established principles of diffusion. The chief resistances to diffusion appear to be at the water-air boundary of the plastron, and from the tracheal endings into the tissues.

6. A comparison of adult and nymphal respiration is given in tabular form, and the necessity for a supplementary mode of respiration in the adult is shown.

7. The development of the plastron in the fifth instar nymph prior to moulting is described, and gas is shown to appear there during the reabsorption of the moulting fluid without the animal having access to the surface. The gas is present before moulting takes place, and before the plastron becomes connected with the tracheal system. The waterproofing of the hairs is probably not a result of secretion of gas into the plastron, but is a necessary condition before gas can replace the moulting fluid there. Moulting takes place beneath the surface of the water.

8. A general theory of the operation of the plastron and its relation to an air store is developed. Since the plastron is capable of withstanding external pressure it can be regarded functionally as a special case of the closed tracheal system. Thus not only the plastron but the whole of the tracheal system containing air must be capable of withstanding the excess pressure Δp . In practice some of this excess pressure may be taken up by the elasticity of the external cuticle, some by the elasticity of the tracheal walls themselves, but a single defect, allowing the volume of the air space to decrease *continuously*, will render the whole system ineffective.

9. It is shown that the plastron in *Aphelocheirus* is unlikely to be rendered ineffective by any environmental condition which the animal is likely to meet, though should the animal descend to great depths the strain on the system would be considerable.

10. The nature of the plastron is shown to be a major factor governing the ecology of the insect. As a result of the development of this method of respiration *Aphelocheirus* has become independent of contact with the air. This in turn has enabled it to inhabit swift-flowing streams without exposure to the danger of being swept away which visits to the surface would entail. The fact that it does not have to carry an air store has thus enabled it to become a true bottom-living predator heavier than water. It can thus occupy an ecological niche not available to any other comparable predatory insect in the adult stage. On the other hand, of course, its method of respiration necessarily restricts it to well-aerated, that is to turbulent, water.

11. An appendix (Appendix II) is supplied in which certain general relations for diffusion in an absorbing medium are deduced, and these are applied to calculations of oxygen diffusion from the tracheal endings.

We are greatly indebted to Prof. D. Keilin, F.R.S., for his kindness in allowing us to use the mercury-sealed gasometer in his department. Without this facility the experiments on the effect of gas mixtures with low oxygen content could not have been carried out.

APPENDIX I

Analysis of diffusion processes in the plastron

BY D. J. CRISP

Let the diffusion constant of oxygen within the hair pile be D , the invasion coefficient be i_0 through the air-water surface, and let x be the co-ordinate measured from the position of the assumed groove at right angles to it. The problem is a two-dimensional one if it is further assumed that conditions are essentially similar along the y -axis.

Let h be the thickness of the plastron, and p be the tension difference between the plastron and the outer medium at a point x .

Consider the element of plastron $dx, dy, ABCD$ (Fig. 3 *b* and *c*).

Across AD oxygen will enter at a rate

$$\frac{dO}{dt} = dx dy i_0 p.$$

Across AB and CD diffusion will occur according to the Fick equation; at AB

$$\left(\frac{dO}{dt}\right)_{AB} = -hD \left(\frac{dp}{dx}\right)_x dy$$

and at CD

$$\left(\frac{dO}{dt}\right)_{CD} = -hD \left(\frac{dp}{dx}\right)_{x+dx} dy.$$

Accumulation within $ABDC$ will therefore be

$$\left(\frac{dO}{dt}\right)_{AB} - \left(\frac{dO}{dt}\right)_{CD} = hD \frac{d^2p}{dx^2} dy dx.$$

Therefore, since

$$\left(\frac{dO}{dt}\right)_{AB} - \left(\frac{dO}{dt}\right)_{CD} - \left(\frac{dO}{dt}\right)_{AD} = 0,$$

therefore

$$\frac{d^2p}{dx^2} - \frac{i_0}{hD} p = 0.$$

At $x = x_1$ (outer boundary) $(dp/dx)_{x_1} = 0$ and at $x = 0$, $(dp/dx)_0$ is maximal and accounts for the whole oxygen intake. If the total intake is q , and $y =$ total groove lengths,

$$\frac{q}{2y} = \left(\frac{dp}{dx}\right)_0 hD.$$

Hence at $x = 0$,

$$\left(\frac{dp}{dx}\right)_0 = \frac{q}{2yhD}.$$

The solution in terms of these boundaries is

$$p = A [Be^{nx} + C \sinh nx], \quad (4)$$

where $n = \sqrt{\frac{i_0}{hD}}$, $A = \left(\frac{dp}{dx}\right)_0 / n$, $B = \frac{1 + e^{2nx_1}}{1 - e^{2nx_1}}$, $C = \frac{e^{nx_1}}{\sinh nx_1}$.

APPENDIX II

Diffusion of oxygen into respiring tissues

By D. J. CRISP

Let the absorption of oxygen be m units per c.c. of tissue and the permeability constant of the medium be P .

(1) *Rectilinear flow*. From a plane at AA' at tension p_1 to any parallel plane at distance t

$$\frac{d^2p}{dx^2} - \frac{m}{P} = 0. \quad (5)$$

Solution for $x=0$, $p=p_1$, $x=t$, $dp/dx=0$ will be

$$p_1 - p = \frac{m}{2P}(2xt - x^2). \quad (6)$$

Hence pressure drop to the plane at distance $x=t$ is

$$p_1 - p_t = \frac{mt^2}{2P}. \quad (7)$$

(2) *Radial flow*. (a) From an inner cylindrical surface of radius r to an outer of radius r_1 ,

$$-\frac{dp}{dr} = \frac{m}{2P}(r_1^2 - r^2)/r. \quad (8)$$

Solution for $p=p_0$, $r=r_0$,

$$p_0 - p = \frac{m}{4P}\left(r_0^2 + 2r_1^2 \log_e \left(\frac{r}{r_0}\right) - r^2\right). \quad (9)$$

Hence pressure drop to a radius r_1 is

$$p_0 - p_1 = \frac{m}{4P}\left(r_0^2 + 2r_1^2 \log_e \left(\frac{r_1}{r_0}\right) - r_1^2\right). \quad (10)$$

(b) From an outer cylindrical surface of radius r , to an inner of radius r_0 ,

$$+\frac{dp}{dr} = \frac{m}{2P}(r^2 - r_0^2)/r. \quad (11)$$

Solution for $r=r_1$, $p=p_1$,

$$p_1 - p = \frac{m}{4P}\left(r_1^2 - r^2 - 2r_0^2 \log_e \left(\frac{r_1}{r}\right)\right). \quad (12)$$

If $r=r_0$,

$$p_1 - p_0 = \frac{m}{4P}\left(r_1^2 - r_0^2 - 2r_0^2 \log_e \left(\frac{r_1}{r_0}\right)\right). \quad (13)$$

And in the limiting case $r_0=0$, $p_1 - p_0 = \frac{mr_1^2}{4P}. \quad (14)$

In both fore- and hindgut and Malpighian tubules the tracheation appears to be limited to a surface network around the gut, from which diffusion must take place through the gut epithelium. In order to find the tension fall in this tissue the diffusion is considered in three stages (Fig. 7).

This treatment, although not rigid, is sufficiently approximate for the present purpose, and gives a value of Δp which is maximal.

(1) Diffusion with absorption through a cylinder of radius $r_1 = l/2$, where l is the mean distance between the tracheoles r_0 is the radius of a tracheole.

From equation (10)
$$\Delta p_1 = \frac{m}{P} \left(\frac{r_0^2}{4} + \frac{r_1^2}{2} \log_e \frac{r_1}{r_0} - \frac{r_1^2}{4} \right).$$

(2) Diffusion without absorption through the annulus bounded by r_0 and r_1 of oxygen required by the region $AA' - BB'$.

For diffusion through the whole annulus:

$$p_0 - p_1 = \frac{q}{2\pi P} \log \frac{r_1}{r_0}. \quad (15)$$

Since diffusion is restricted to half the annulus we shall assume twice the fall in tension,

$$\Delta p_2 = \frac{q}{\pi P} \log \frac{r_1}{r_0}, \quad (16)$$

and $q = 2m(t - r_1)r_1$, where t is the thickness of the gut. Therefore

$$\Delta p_2 = \frac{2m}{\pi P} \left[r_1(t - r_1) \log \left(\frac{r_1}{r_0} \right) \right].$$

(3) Diffusion from AA' to BB' assuming AA' to be at uniform tension. From equation (7) above,

$$\Delta p_3 = \frac{m}{P} \left[\frac{(t - r_1)^2}{2} \right].$$

Hence total pressure drop

$$= \Delta p_1 + \Delta p_2 + \Delta p_3 = \frac{m}{P} \left[\frac{r_0^2}{4} + \frac{r_1^2}{4} \left(2 \log \frac{r_1}{r_0} - 1 \right) + \frac{(t - r_1)^2}{2} + \frac{2}{\pi} r_1(t - r_1) \log \frac{r_1}{r_0} \right]. \quad (17)$$

The striated musculature is arranged in bundles on the outside of which there exists a close network of tracheoles, and a few can be seen to pass inside.

It is very difficult to determine the distribution at all exactly, but we have taken two extreme possibilities.

(a) The tracheal supply is confined to an envelope from which diffusion occurs to the centre of the muscle bundle (Fig. 8). A treatment similar to that applied to the gut based on equations (10), (14) and (16) gives

$$\Delta p = \left[\frac{m}{P} \frac{r_0}{4} + \frac{r_1^2}{4} \left(2 \log \frac{r_1}{r_0} - 1 \right) + \frac{(R - r_1)^2}{4} + \frac{r_1(R - r_1)}{\pi} \log \frac{r_1}{r_0} \right]. \quad (18)$$

(b) The tracheae interpenetrate the whole bundle, their density being equal to that observed at the surface. This value may be found from equation (10).

REFERENCES

- BIRGE, E. & JUDAY, C. (1911). *Wisconsin Geol. Nat. Hist. Survey*, **22**, 52.
BUTCHER, R. W., PENTELow, F. T. K. & WOODLEY, J. W. A. (1930). *Int. Rev. Hydrobiol.* **24**, 47.
BUTCHER, R. W., PENTELow, F. T. K. & WOODLEY, J. W. A. (1937). *Biochem. J.* **21**, 1424.
CRISP, D. J. & THORPE, W. H. (1947). *J. Exp. Biol.* **24**, 304.
EGE, R. (1918). *Z. allg. Physiol.* **17**, 81.
FOX, H. M. & WINGFIELD, C. A. (1938). *J. Exp. Biol.* **15**, 437.
FRAENKEL, G. & HERFORD, G. V. B. (1938). *J. Exp. Biol.* **15**, 266.
KOCH, H. J. A. (1936). Recherches sur la Physiologie du Système Trachéen Clos. Extrait des *Mémoires publiés par l'Acad. royale de Belgique* (Classe des Sci.), **16**, 1.
KROGH, A. (1908). *Skand. Arch. Physiol.* **20**, 259.
KROGH, A. (1910). *Skand. Arch. Physiol.* **23**, 224.
KROGH, A. (1919). *J. Physiol.* **52**, 391.
KROGH, A. (1920). *Arch. ges. Physiol.* **179**, 95.
MALOEUF, N. S. R. (1936). *J. Exp. Zool.* **74**, 323.
SCHULMAN, J. H. & TEORELL, T. (1938). *Trans. Faraday Soc.* **34**, 1337.
THORPE, W. H. (1932). *Proc. Roy. Soc. B*, **109**, 450.
THORPE, W. H. & CRISP, D. J. (1947). *J. Exp. Biol.* **24**, 227.
WIGGLESWORTH, V. B. (1930). *Proc. Roy. Soc. B*, **106**, 229.
WIGGLESWORTH, V. B. (1933). *Quart. J. Micr. Sci.* **76**, 286.

A METAL MICRO-RESPIROMETER OF THE BARCROFT TYPE SUITABLE FOR SMALL INSECTS AND OTHER ANIMALS

By D. J. CRISP

Department of Colloid Science, University of Cambridge

AND W. H. THORPE

Sub-Department of Entomology, Zoological Department, University of Cambridge

(Received 10 December 1946)

(With Two Text-figures)

METAL BARCROFT RESPIROMETER

The metal Barcroft respirometer is an accurate and rapidly operated micro-respirometer, suitable for small insects and other animals which can be maintained in an aerial environment for the period of experiment, and is particularly suitable for experiments in which different gas mixtures are employed. It is based on the principle of the differential (Barcroft) type of manometer, but its chief advantages are its small size and construction from a solid brass block. In consequence, the temperature surroundings of the two chambers are kept uniform by the high conductivity of the metal, and gaseous diffusion is very rapid throughout.

Into a solid brass block *ABCD* are drilled two chambers, the experimental chamber (1) being 1 cm. in diameter and 1 cm. deep, the control chamber (2) being 2 cm. in diameter and 1.75 cm. deep. The chambers taper at the base and are continued as fine capillaries 1.0 sq.mm. in cross-section (*EF* and *GH*) to the base of the block, where they terminate in small brass collars. A bent capillary tube *R* of cross-section 0.2 mm., graduated in cm., containing the manometer fluid, is fitted directly into these collars and sealed with picene wax. The chambers 1 and 2 each communicate with the outside by means of fine capillaries *JK*, *LM*, drilled in the metal and terminating in collars, and intercommunicate by another capillary *PQ*. These capillaries may be opened and closed by taps *U*, *V*, *W*, as shown. The chambers are closed above by glass plates (*S*) cut from microscope slides, which are pressed tightly down on to a thin layer of Apeizon H.V. grease smeared on the surface of the block. If the surface of the block is polished, it is possible to tell by inspection when the chambers are completely sealed off, since any small leak is apparent as an air channel in the grease. We find this type of seal less troublesome than the greased ground-glass join of the standard Barcroft. Not only is it easy to ensure freedom from leaks, but there is also no tendency for the grease to be squeezed out, causing an alteration in the volume of the chamber. In order to reduce any tendency to corrosion, the insides of the chambers are tinned.

Each chamber is furnished with a small hole (O) into which is fitted a glass tube containing a solution of potash (10%) and a piece of starch-free filter-paper.* A gauze frame *T* separates the insect from contact with the potash.

The chief disadvantage of the apparatus is the relatively large amount of dead space involved in the capillaries. This is unavoidable, since if the capillaries are made any narrower than 1 sq.mm. in cross-section they are liable to blockage by condensation, and further, the taps do not operate successfully. These in fact constitute the chief weakness in the design, and great care should be taken to ensure that the tapers are accurately machined. On the other hand, the dead spaces, unlike those of the standard Barcroft manometer, are separated from the chambers by very short diffusion paths, and may be treated as parts of the chambers. The difference in volume of the chambers serves to increase the sensitivity of the apparatus.

CALIBRATION

The apparatus is most conveniently calibrated by calculation from its dimensions. We give below a summary of this calculation:

Volume of experimental chamber (1)

Depth to edge of conical part	= 0.7 cm.
Total depth	= 1.0 cm.
Radius	= 0.5 cm.
Length of capillary to base of block	= 3.0 cm.
Length of capillary to outer tap	= 1.0 cm.
Length of capillary to inner tap	= 0.8 cm.
Mean length of space above manometer fluid	= 3.0 cm.
Cross-sectional area of capillaries in metal	= 0.01 sq.cm.
Cross-sectional area of manometer capillary	= 0.002 sq.cm.
Total volume = $\pi \times (0.5)^2 (0.7 + 0.3/3) + 0.01 (3.0 + 1.0 + 0.8) + 0.002 \times 3.0$ c.c.	
	= 0.628 + 0.048 + 0.006
	= 0.682 c.c.

Volume of gauze = 0.050 c.c. (by weighing).

Volume of insect (approx.) 0.02 c.c.

Net volume of chamber (1) = 612 cu.mm. = V_g in Dixon's formula.

Similarly, for chamber (2), the volume is found to be 4710 cu.mm. = V_g' .

Density of oil P_1 = 0.79 g./c.c.

Manometer vertical θ = 0

Volume of fluid V_f = 0.100 c.c.

Cross-section of manometer capillary = 0.207 sq.mm.

(by weight of mercury thread)

Temperature = 20° C.

By Dixon's (1943) formula, uptake in cu.mm.,

$$x = h \left(1 + \frac{0.207 \times 13,110}{2 \times 4710} \right) \left(\frac{(612 \times 273/293 + 100 \times 0.03)}{13,110} + \frac{0.207}{2} \times \frac{273}{293} \right) = 0.181h.$$

* Provided that the apparatus is kept at the small dimensions described the filter paper can be omitted with advantage.

The measurements were carried out by observation with a microscope carrying an eyepiece scale focused on one arm of the manometer. If this movement is h' mm., $x = 0.362h$.

Calibration of eyepiece scale $9.8 \text{ units} = 1 \text{ mm.}$

Hence uptake in cu.mm./hr. $= \frac{60 \times 0.362}{9.8} \cdot n = 2.22n$, when n is movement of column in eyepiece units per minute.

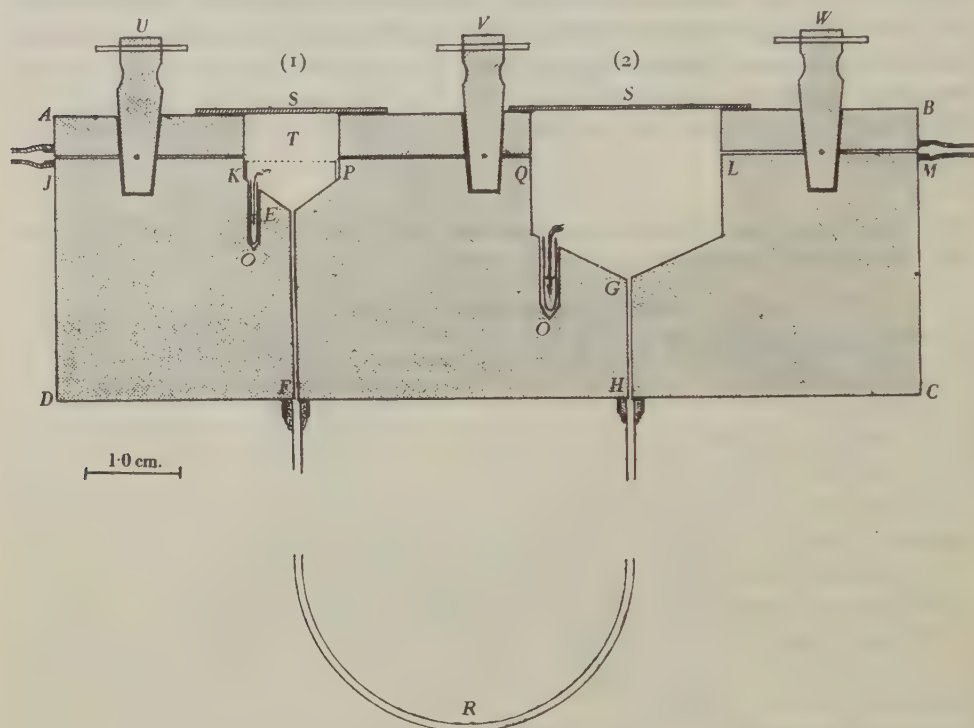


Fig. 1. Diagrammatic section of micro-respirometer. (For explanation see text.)

MANIPULATION

Before fixing the capillary in position the apparatus is cleaned by means of a volatile solvent (ether) which is removed by passing air through the apparatus. The capillary tube of the manometer is cleaned by means of chrome sulphuric acid and distilled ether; any dirt renders the column of paraffin sticky and greatly reduces the accuracy of the apparatus. When filled to a convenient level with paraffin the manometer tube is sealed into position. The apparatus can then be used for an indefinite number of experiments.

Before each experiment the potash in the small tubes and the filter-paper is removed and replaced with fresh and the cover-plates are cleaned and regreased. The gauze T is replaced and the animal inserted in the left-hand chamber. If it is desired to employ a synthetic gas mixture, all the taps are opened and the mixture

passed slowly through. This is best done in two stages, about 70 c.c. being passed at a time, in order to ensure complete exchange in the dead spaces. After passage of gas, taps *U* and *W* are closed, and the apparatus immersed in a thermostat up to the level of the side collars. The level in the manometer limbs should then be equal. When the gas mixture has equilibrated sufficiently (this usually only takes about 2 min.) the centre tap *V* is closed and readings are taken through the microscope. If it is desired to test the efficiency of the taps, the finger can be laid lightly on one of the cover-plates. This will cause a sudden fall of the fluid in this limb, followed by a slower recovery which, however, is complete after about 60 sec., owing to the very rapid temperature equilibration of the apparatus. If the tap *V* is momentarily opened and closed after touching one of the cover-plates, a persistent difference in the manometer levels should be caused. Sustained pressure at *J* or *M* should have no effect on the manometer level. Readings are then taken at intervals and the animal may be observed through the cover-plate.

The thermostating arrangements were found not to be critical on account of the high thermal conductivity of the apparatus. The most suitable arrangement was found to be a large tank of water in a constant-temperature room, but the apparatus worked reasonably well if immersed in a large thermostating device. It was found to be better to have a bath at uniform though steadily changing temperature than one at constant temperature but fluctuating suddenly between narrow limits. In this connexion it should be pointed out that the difference in size of the two chambers does not matter so long as they change equally in temperature, since the coefficient of increase in pressure with temperature is the same in each. If, however, their rate of change were uneven (as would be the case with glass bulbs of low thermal conductivity) the apparatus would become highly temperature sensitive. The low conductivity of the cover-plates is, however, advantageous when the apparatus is not fully immersed, as it damps down any effect of local draught or radiation.

SENSITIVITY AND ACCURACY

The theoretical sensitivity of this apparatus is very high. The scale eyepiece can be read easily to one scale division corresponding to a change in volume of the experimental chamber of 0.037 cu.mm. The comparable sensitivity of the standard Barcroft with a constant of 3-4 and an accuracy of reading of half a millimetre division would be about 3-4 cu.mm. Hence the sensitivity is increased approximately a hundredfold.

The accuracy is limited by two errors. First, by small errors in calibration, and secondly, by slow changes in volume which occur over a long period of time. The errors in calibration are relatively greater than those encountered with the standard Barcroft, owing to the small size of the experimental chamber, but it is unlikely that they exceed 4% in all. The second source of error is the more serious. If the apparatus is set up with the experimental chamber empty, steady small changes are sometimes observed over a long period of time. Table 1 gives the results of some typical control experiments, and it will be seen that the error does not exceed at most 8 div./hr., corresponding to an uptake of less than 0.3 cu.mm./hr., and is usually much less.

The source of this error has not been traced. It is not due to leaks as it is independent of any pressure difference indicated by the manometer. As it is always positive, corresponding to uptake of gas, it may possibly be due to points of corrosion in the apparatus, but no corrosion could be found on examination and the rate of change was somewhat variable. The error is, however, comparable to that caused by the difference in uptake by the small pieces of filter-paper used, and is not therefore very serious.

Table 1

Duration of exp. (hr.)	Rate of change of reading (units/hr.)	Apparent uptake (cu.mm./hr.)
3	1	0.04
25	7	0.26
7	0	0.0
22	2	0.08

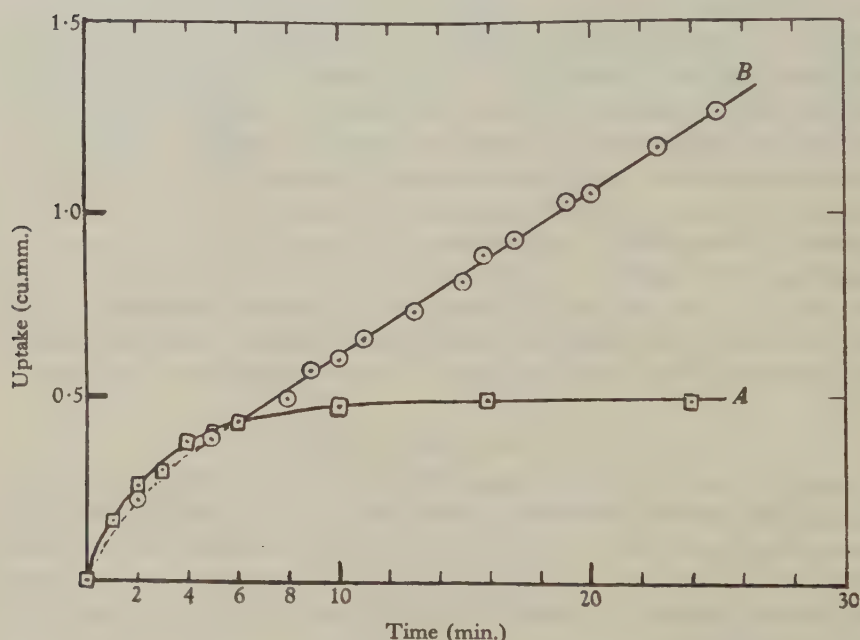


Fig. 2. Oxygen uptake curves. A. Control experiment with respirometer empty. Note apparent initial uptake due to vapour-pressure effect. B. Experiment with single *Aphelocheirus*. Note apparently greater uptake initially.

The initial equilibration of the apparatus is very rapid. After placing in the water-bath, about 10–15 min. only are required for temperature equilibration, while after small temperature changes caused by handling or passing in a gas mixture the apparatus requires less than 5 min. to become steady. It has been observed that vapour-pressure equilibration takes rather longer. When a damp insect is inserted and the chamber closed, the vapour pressure is close to that of liquid water.

After a short time vapour distils on to the potash-covered filter-paper until the vapour pressure becomes uniform. During this interval the vapour pressure in the experimental chamber is falling, and this gives the impression of an increased initial absorption. This effect is shown in Fig. 2. The effect is still there when no insect is inserted, as the air initially in the chamber is drawn from the immediate surroundings of the water-bath and is saturated. The effect is reduced when more dilute potash is employed, but as long as readings are not taken for about 15 min., when the uptake becomes steady, no error is involved.

When the apparatus has equilibrated it is singularly free from oscillations in the manometer due to temperature changes in the bath or surroundings, so that it is possible to obtain reliable rates of respiration over a very short time interval. For instance, Fig. 2 also gives the results of an experiment where the rate of respiration was 3.5 cu.mm./hr., the whole determination being made in less than 30 min.

This apparatus has been employed by the authors to investigate the respiration of the hemipteron *Aphelocheirus*, and the results of this investigation are now published (Thorpe & Crisp, 1947).

SUMMARY

A Barcroft-type micro-respirometer, made of metal, is described. It is particularly suitable for rapid determination of the respiratory rate of small insects or other animals which can be maintained in air during the course of the experiment. It is especially convenient for experiments in which it is necessary to employ different gas mixtures. Sensitivity is approximately 100 times that of the standard glass Barcroft apparatus. The possible sources of error are considered.

We are indebted to Dr H. Laser for helpful discussion and for his readiness to give us the benefit of his special knowledge of manometric methods.

REFERENCES

- DIXON, M. (1943). *Manometric Methods*, 2nd ed. Cambridge.
THORPE, W. H. & CRISP, D. J. (1947). *J. Exp. Biol.* **24**, 270.

STUDIES ON PLASTRON RESPIRATION

III. THE ORIENTATION RESPONSES OF *APHELOCHEIRUS* [HEMIPTERA, APHELOCHEIRIDAE (NAUCORIDAE)] IN RELATION TO PLASTRON RESPIRATION; TOGETHER WITH AN ACCOUNT OF SPECIALIZED PRESSURE RECEPTORS IN AQUATIC INSECTS.

BY W. H. THORPE

Sub-Department of Entomology, Zoological Department, University of Cambridge

AND D. J. CRISP

Department of Colloid Science, University of Cambridge

(Received 3 February 1947)

(With Six Text-figures)

1. INTRODUCTION

In the first paper of this series (Thorpe & Crisp, 1947, later referred to as Part I) it was shown that the active predatory bug *Aphelocheirus aestivalis*, which inhabits the moderately swiftly flowing parts of rivers and streams, provides one of the most perfect instances of respiration by means of a gaseous plastron. This method enables the animal to obtain all its oxygen from that dissolved in the water by means of an extremely thin film of gas, of constant but negligible volume, into which specially modified spiracles open. This film of gas acts as a gill, and is held in position by a pile of exceedingly minute hairs extending over the greater part of the body surface. The structure, arrangement and size of these hairs is such that they are unwettable by any surface forces and any degree of hydrostatic pressure, either separately or combined, to which they are likely to be subjected in the normal environment of the animal. Consequently there is no danger of the air film being lost and similarly no need for the animal ever to come to the surface. Since *Aphelocheirus* carries no air store or other appreciable volume of air with it, in contradistinction to other adult aquatic insects it is heavier than water. This, in combination with its powers of swimming and its ability to cling tenaciously to stones and vegetation, has enabled it to become a true bottom-living form.

Although *Aphelocheirus* is mechanically well adapted to resist increase of pressure up to 3 atm., it must, on account of its dependence on the oxygen content of the water, be safeguarded against sudden or uncontrolled descent into the deeper parts of the river where it might be trapped in a region of low oxygen tension. It therefore requires some mechanism to prevent its being swept by the current into stretches of the river where, for this or other reasons, the water is unsuitable. It is the purpose of this paper to consider the orientation mechanisms of *Aphelocheirus* in relation to its respiratory needs—that is, in effect, the main responses which ensure its ability to maintain itself in regions where the medium is suitable for its life and development.

2. REACTIONS TO LIGHT

(i) *Light compass reaction*

The well-developed compound eyes are presumably capable of the light compass reaction and almost certainly of rudimentary form sight. This sense will therefore enable the insect to maintain position and direction in daylight when clinging to the substratum or when swimming in close proximity to objects.

(ii) *Dorsal light response*

That *Aphelocheirus* has a well-developed 'dorsal light response' can be shown by placing the insects in a dark room in a glass tank which can be lit from beneath by a 40 W. bulb. Directly the bugs are exposed to bottom lighting there is a marked tendency for them, if at all active, to swim on their backs. If they are stationary on the bottom or if they finally settle down they tend to remain the right way up, or at any rate soon to right themselves in spite of bottom lighting. This response to reversed lighting tends to fade as soon as they grow more accustomed to the tank, and consequently less active. Also, after some period of conflict between the dorsal light response and the thigmotactic response to the bottom, the latter tends to get the upper hand and the light is disregarded to some extent. The same response is found in fifth instar nymphs, provided again that they are in an active state. *Aphelocheirus* then has the ability to direct its swimming in the vertical axis by reference to the direction of incident light.

3. REACTION TO PRESSURE

(i) *The paired abdominal sense organs*

A hitherto unrecognized pressure receptor is to be found in the form of a pair of oval sensory plates situated on the sterna of abdominal segment 2 (Fig. 1). They show a well-developed nerve supply. Comparison of their appearance in section (Fig. 2) with the somewhat similar sense organs found in *Nepa* and which Baunacke (1912) had decided were pressure receptors, gives one good reason to assume at the outset that this is also their function in *Aphelocheirus*. The organs in *Aphelocheirus* consist of shallow depressions in the cuticle, these depressions being devoid of the typical plastron hairs of the body surface. Instead of plastron hairs the depressed surface is covered by a large number (approximately 60,000 per sq.mm.) of very much larger hydrofuge hairs, all pointing backwards and inclined at an angle of approximately 30° to the surface. In among these hairs are dispersed at intervals somewhat more thinly walled hairs, each supplied with a sense cell, the proximal processes of the cells which penetrate these sensory hairs together making up the nerve. The structure of these sensory hairs is in most respects characteristic of the tactile type of sensillum and the mode of action of the organs is assumed to be as follows: When the pressure is increased, the air film on the sense organ will be compressed, and since the hairs are strongly hydrofuge they themselves will be pressed down, thus moving the sensory hairs at the same time. When the pressure is decreased the air film will expand and the hydrofuge hairs will be enabled by their elastic tension to resume their former position.

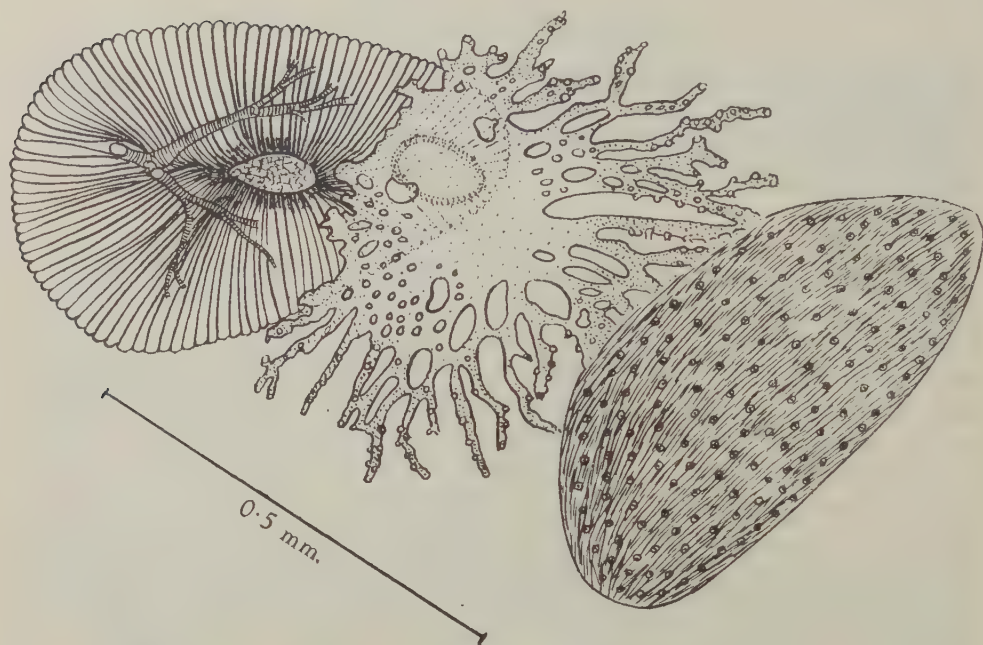


Fig. 1. Left spiracular 'rosette' on second abdominal segment of *Aphelocheirus* with the adjacent specific organ of pressure sense and the collapsible air sac. Ventral view of whole mount by transmitted light; cuticle rendered transparent.

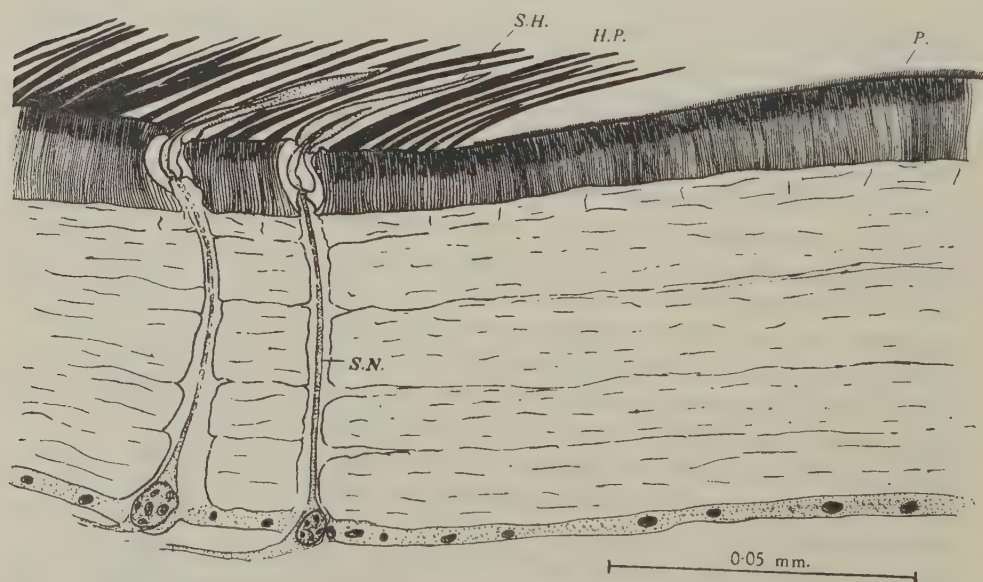


Fig. 2. Section through edge of organ of pressure sense of *Aphelocheirus*. H.P. = hair pile; S.H. = sensory hairs (trichoid sensilla); P. = plastron hair pile; S.N. = sensory nerve.

The proximal margin of the sense organ runs right across and thus truncates the spiracular 'rosette', so that the air film on the sense organ is in intimate contact with the tracheal system via the minute spiracular openings. The two spiracles concerned differ from all the other spiracles in that they each open into a small air sac which itself gives off a fine tracheal branch to the main tracheal trunk as well as finer branches supplying nearby tissues. The air sac is (Figs. 1, 3) of remarkable appearance, its walls being folded and ribbed in a peculiar concertina-like manner such as might allow it a possibility of undergoing great changes in volume with changing hydrostatic pressure. Under a high power of the microscope the air sac resembles to some extent the air bladders of the larvae of *Corethra* and, just as in that insect,

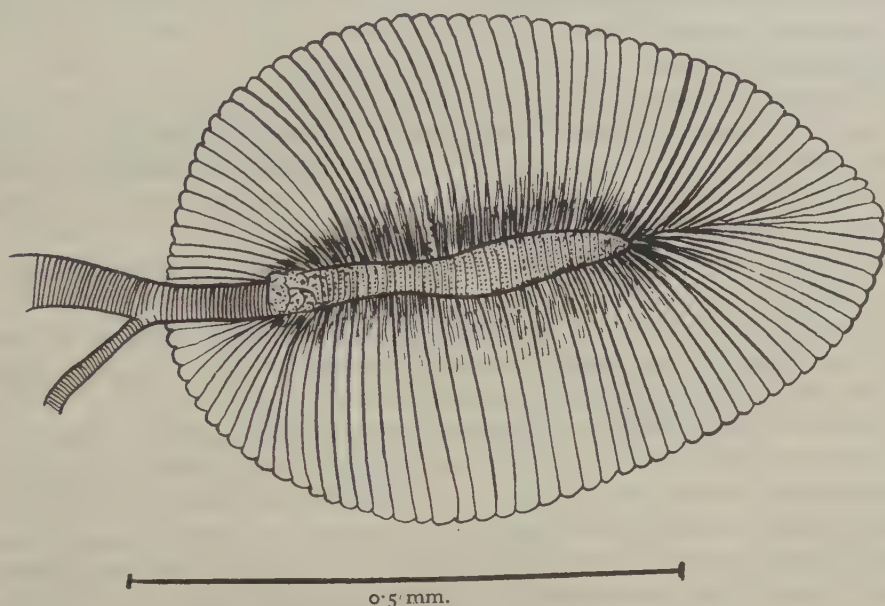


Fig. 3. Collapsible air sac of *Aphelocheirus*, dorsal view.

we have been unable to find any muscles or contractile fibres. If they were indeed present, it should be easy to detect them with the polarizing microscope. Dissection failed to reveal any nerve supply to the structure. It has not been possible to secure any direct evidence throwing light on the possible function of this air sac. Its resemblance to the air sac of *Corethra* larva is remarkable, but its volume is far too small—not more than one-fiftieth of the total tracheal volume—for it to have any function as a hydrostatic organ; nor is a hydrostatic organ required. The possibility that it acts as a volume control, maintaining at a constant level the position of the gas-water interface on the special organ of pressure sense with which it is in such close relation, seems ruled out by the fact that it is in open connexion with the main tracheal trunk via a fine trachea without any intermediate valve. It does, however, provide a very small adjustable region in a tracheal system which as a whole must be highly resistant to compression, and its probable function will be considered

below when the exact mode of action of the sense organ is being discussed. Before leaving this subject it should be recalled that a small air sac, but without the peculiar folded structure of the present example, is found in other aquatic bugs (e.g. *Nepa* and *Naucoris*) in much the same position relative to the second abdominal spiracle but not associated with any specific sense organ.

(ii) *Pressure responses*

The types of response to pressure and to low-frequency pressure changes in any aquatic animal may conveniently be classified in one of three categories:

(a) Response to local low-frequency pressure changes due to currents, turbulence and eddies in the surrounding medium.

(b) Response to changes in total pressure caused by changes in the depth of the animal below the surface.

(c) Response to pressure differences on the body surface resulting from the position of the animal in relation to the direction of gravity. This type of response is analogous to that given by a statocyst but is brought about through the small variation in pressure of the medium with depth over the body surface or between specialized receptors, and not a direct response to gravity.

The ability of *Aphelocheirus* to respond to each of these in turn will now be discussed.

(a) The considerable equipment of trichoid tactile sensilla possessed by both nymph and adult, together with the antennae, are likely to provide sense organs indicating the presence and direction of local eddies and currents.

(b) *Aphelocheirus* adults have a characteristic behaviour response to changes in pressure which will tend to keep them from going too deep. When placed in a pressure jar they show a characteristic movement with each increase in pressure. This movement consists of a sudden jerky swim upwards, often reaching to the surface. Even if not fully manifested owing to the bugs being in a sluggish condition, it is rare for the pressure to be increased without the animals giving at least a single kick with the hind legs. That this response is due directly to pressure and not to rapid sensitivity to solution of oxygen from the tracheal system consequent upon the water being less than at saturation at the imposed additional pressure, is shown by the fact that the response remains both when the water is saturated with oxygen and when the insects are made comatose by being kept in freshly boiled water. A sudden increase in pressure will at once produce momentary signs of activity even under these conditions. A reduction of pressure, on the other hand, produces no response of any kind under any conditions. The nymphs, on the other hand, give no clear and definite response to increases in pressure unless these are very sharp and sudden. The most which is obtained with increases of up to 70 cm. mercury is a slight general increase in activity.

It was also found that adults gave the same response to pressure when exposed to a diffuse red light in the dark room as they did under ordinary conditions of light, showing that the upward swimming was not dependent for guidance on the dorsal light reflex.

It can readily be shown, as described below, that the specialized receptors on the abdomen, which have been described above (§ 3 (i)), are in part responsible for the sensitivity to pressure increment. Various methods were tried for eliminating these sense organs, such as wetting them with a drop of 'Nujol' or sealing them with paraffin wax. Finally, it was found that the best method was to touch them with a platinum micro-cautery heated to a point just sufficient to destroy them rapidly. It was curious that this treatment seemed to result in far less shock than did the treatment with paraffin wax or 'Nujol'. Bugs treated in this way were observed in a pressure control tank. It was found that bugs with both sense organs cauterized showed a general diminution in sensitivity to vibration of the substratum, but that the characteristic jerk of the swimming legs in response to abrupt pressure increase, while less evident, was by no means eliminated. This suggests that major pressure increases can be appreciated by some other organs—probably by the proprioceptor sense organs of the cuticle. It must be admitted that elimination of the sense organs would probably make directed upward swimming more difficult, and this might account for some lack of response even if the pressure increment were appreciated. Since, however, the response is diminished appreciably even in the light which has been shown to be effective in directing movement, it is reasonable to assume that the apparent loss in sensitivity is real and is due to the extirpation of the sense organs.

(c) It is readily observed that adults are capable of directed swimming on an even keel in dull red illumination to which they are insensitive. This involves the appreciation of position in relation to gravity in order to maintain stability during swimming, and it was thought probable that the paired abdominal sense organs might be responsible for this. Intact bugs (controls) and others from which the sense organs had been eliminated were tested for their ability to make direct downward swims (as distinct from passive uncontrolled sinking) in a large tank containing water 10 in. deep, at atmospheric pressure and a temperature of 16° C. Experiments were carried out in the dark room with a diffuse Wratten safelight on one side of the tank. When light was required from above, a 40 W. lamp was used 7 in. above the water surface.

The results of these experiments are set forth in Tables 1-4. Table 1 gives the times taken by a number of bugs to reach the bottom of the tank after being placed just below the surface. In order to overcome individual variations seven operated and ten control individuals were used, and a mean taken from the times of twenty trials. Applying the *t* test of significance to the means it is found that in the dark the cauterized individuals take much longer than the controls with a high level of significance (between 0.01 and 0.001). The ability to swim in the light is also affected, and here again the significance level lies between 0.01 and 0.001. It is also likely that even normal bugs will swim more efficiently in the light than in the dark (level 0.05). This points to the conclusion that in the light a directed swim downwards or upwards can be accomplished by means of a directional light response. In the dark only those bugs with the sense organ intact are sensitive to small pressure changes and so swim directly to the bottom; those with the sense organ cauterized are badly disorientated. In order to assess this factor, experiments were repeated

under the same conditions, except that this time the number of turns taken by the bugs during the swim to the bottom was counted, all experiments being done in a diffuse red light. It will be seen that here again there is a marked difference as between the experimental animals and the controls in the number of turns taken.

The significance of the results in Exps. I and II in Table 2 may be shown as follows: The time taken for a turn is at most 1 and at least $\frac{1}{4}$ sec. We may divide up the total time (adding both sets of results) into periods of 1 sec. or into $\frac{1}{4}$ sec. intervals. Each interval may be considered as a trial during which a turn may or may not occur. In this way 2×2 contingency tables are obtained representing the two extremes (Table 2A).

The value of χ^2 for the 0.05 level of significance for 1 degree of freedom is 3.84. It therefore follows that even after allowing for the longer time taken to reach the bottom by the operated bugs, the number of turns in unit time (rate of turning, Table 2) is slightly but significantly higher in the operated animals. It appears that the difference between the time taken by the cauterized and the control bugs to reach the bottom is not much, if at all, due to different speeds of swimming, but is an expression of the fact that whereas intact bugs usually swim straight downwards in a purposive manner, more turns and gyrations are taken by the cauterized bugs.

From Table 2 (Exp. III) it will be seen that fifth instar nymphs behaved rather like cauterized adults; although they turn less often they have little or no power of directed swimming in the absence of light. They tend to sink passively in both light and darkness; the proportion of 'sinkers', however, appears higher in the dark, but the difference is scarcely significant (0.1-0.05 level).

Some experiments were then performed in which the sense organ of one side only was treated. These experiments were set up as previously in diffuse red light. The general impression from preliminary observations with bugs with the right sense organ only destroyed was that they dived with a right-handed spiral, and those with the left destroyed with a left-handed spiral, but it was extremely difficult to follow accurately. Careful observations were therefore undertaken using a more precise classification of behaviour, and these are summarized in Tables 3 and 4. The connotation of the letters used in the tables is as follows: F, falling passively to bottom; S, swims straight to bottom; D, dorsal surface uppermost; U, swims upwards; R, right-hand spiral; L, left-hand spiral; RL, right- and left-handed spirals alternating; T, turns over in tumbling fashion; H, horizontal swimming; V, ventral surface uppermost.

From Table 3, which gives the result of a typical experiment, it will be seen that with both sense organs cauterized the direction of turning is random. In contrast to this, the turning of insects in which the left sense organ only is cauterized and the right sense organ only is cauterized is markedly directional.

The results in Table 4 were analysed statistically and significant differences are given in heavy type.

It will be seen that the result of unilateral cautery of the sense organ is to produce a spin towards the same side, to reduce the ability to swim straight to the bottom (S), and to increase the total number of turns (R and L) taken. When both organs are

Table 1. *Time taken by bugs to reach bottom after being placed in the water just below surface. 16° C.*

Cauterized bugs (7 individuals)				Controls intact (10 individuals)			
Time in sec. 20 swims				Time in sec. 20 swims			
In dark				In dark			
12,	10,	3,	10	3,	2,	3,	8
24,	3,	6,	3½	3,	4,	2½,	3
54,	4,	13,	13	2½,	2½,	2,	2
20,	6,	8,	6½	4,	2,	3,	5
8½,	7½,	10,	8	7,	6,	3,	3
Total 230 sec.				Total 70½ sec.			
Av. duration of swim: 11.5 sec.				Av. duration of swim: 3.5 sec.			
In light				In light			
2½,	3½,	2½,	3	4,	2,	2,	3½
3,	9,	4,	3½	4,	3,	3,	4½
3,	4,	3,	6	2,	3,	3,	1
2,	6,	4,	2	3½,	2,	2,	1½
6,	5,	6,	2	3,	2,	2,	1½
Total 80 sec.				Total 52½ sec.			
Av. duration of swim: 4 sec.				Av. duration of swim: 2.6 sec.			

(For explanation see text.)

TABLE 2. *Observation on number of turns taken by bugs during swim to bottom. Exps. I and II, adults. Exp. III, nymphs. All experiments in dark (except as stated in Exp. III)*

Exp. I. 16° C.

Cauterized bugs (7 individuals).

77 turns in 20 runs.

Total time: 153 sec.

Average time: 7.6 sec.

Rate of turning: 1 in 2 sec.

Exp. II.

Cauterized bugs (7 individuals).

76 turns in 20 runs.

(N.B. five runs were largely or entirely passive 'sinks'.)

Total time: 145 sec.

Average time: 7.2 sec.

Rate of turning: 1 in 1.9 sec.

Exp. III. Using four intact fifth instar nymphs as comparison. 12° C.

Dark.

20 runs.

17 sinkers.

1.1 turns in remaining 3 runs.

Total time: 237 sec.

Average time: 11.8 sec.

Exp. IA.

Controls. Intact bugs (10 individuals).

(N.B. These bugs had been in an 'experimental' tank in dark for 4 hr. and were probably somewhat tired.)

40 turns in 20 runs.

Total time: 117 sec.

Average time: 5.8 sec.

Rate of turning: 1 in 2.9 sec.

Exp. IIA. Controls. (These bugs had been rested in a 'furnished' tank and were nearly back to normal.)

37 turns in 20 runs.

(No 'sinks'.)

Total time: 93 sec.

Average time: 4.7 sec.

Rate of turning: 1 in 2.5 sec.

Light.

20 runs.

11 sinkers.

5 turns in remaining 9 runs.

Total time: 140 sec.

Average time: 7 sec.

TABLE 2 A.

	Turns	Non-turns	Total trials	χ^2
(a) Each trial 1 sec.: Operated	153	145	298	10.0
Control	77	133	210	
(b) Each trial ½ sec.: Operated	153	1039	1192	6.2
Control	77	763	840	

cauterized the total number of turns is reduced, but instead of swimming directly to the bottom, all sense of depth appears to have been lost, the number of horizontal (H) and upward (U) swims being possibly higher, and the tendency to sink passively (F) is significantly greater. *Aphelocheirus* adults therefore can control the direction of their swimming in relation to the vertical axis either by the light compass response or by very sensitive specific pressure receptors. The nymphs, on the other hand, lack the specific receptors and are therefore dependent solely upon the light compass response.

TABLE 3. *Observations on number of turns taken by bugs during swim to bottom when both sense organs cauterized (all experiments in dark). 16° C.*

1. FV	11. HD, U, LD, RV
2. FV, HD, R, and LV	12. T, S, T
3. FV, LD	13. T, HD, LD, HD, V, LV
4. FV	14. H, U, T, FD, T
5. FV, RV	15. U, FD, HD, S
6. U, HD, FH, T	16. S, T
7. FV, LV, RV	17. RS, LS, U, HD, HV, U, T, HD, T
8. HD, RD, FV	18. FV
9. U, FD, HD, S	19. LS, S
10. HD, FD, T, FV	20. HD, U, HV, U, LS, U, HD

(For explanation of lettering see text.)

TABLE 4. *Behaviour of (a) controls, (b) left sense organ cauterized, (c) right sense organ cauterized, (d) both sense organs cauterized. 16° C.*

Two specimens of each except in (c) (only 1) allowed to drop 20 times in deep tank. Percentage time occupied in each type of behaviour is given.

	(a) Controls	(b) Left cauterized	(c) Right cauterized	(d) Both sense organs cauterized
S	45.2	27.6	20.3	9.8
H	10.2	1.0	10.8	15.3
U	2.7	2.3	4.9	10.5
F	10.6	17.6	11.7	31.8
T	8.5	13.8	5.8	12.0
R	12.3	3.7	38.8	8.9
L	10.5	34.0	7.7	11.7
	22.8	37.7	46.5	20.6

(For explanation of lettering see text.)

4. REACTIONS TO OXYGEN CONTENT OF MEDIUM

As recorded in an earlier paper (Part I), *Aphelocheirus* nymphs and adults show a rapid response to lack of oxygen in the surrounding medium. If placed in boiled distilled water they exhibit spasmodic but powerful upward swims to the surface, then display a typical attitude characteristic of asphyxia, and finally become comatose. The initial behaviour is similar to that described above as a result of sudden externally applied pressure, and it appeared possible that the organs of pressure sense might be responsible for both reactions.

In any organ of pressure sense, the movable sensory member (drum, membrane, hydrofuge hairs, etc.) should ideally have a definite position corresponding to a given external pressure. Moreover, it is essential that the medium internal to this member should be compressible. In its simplest form, therefore, the organ of pressure sense will be open to the environment on one side and to an internal gas-

filled space on the other. The correct functioning of the organ will now depend upon the constancy of pressure which can be maintained within the internal space. As this space must be filled with gas in equilibrium with or actively secreted by the surrounding tissues, its pressure will only remain constant within the limits either of the constancy of gas tension in the tissues or the regulatory powers of the secretory system. It therefore follows that to achieve any absolute pressure sense acting over long periods will be a very difficult problem for the animal.

On the other hand, a pressure sense acting over a short period of time will require only that the pressure changes in the internal space should be slow. In the extreme case of organs of hearing the pressure changes are so rapid that the internal space may also open into the atmosphere so that the pressure on both sides of the moving member is equal except for high-frequency vibration.

In *Aphelocheirus* the internal space of the organ of pressure sense is continuous with the plastron, and this in turn with the air in the tracheal system. It is essential therefore that the air in the tracheal system should remain at a constant pressure, otherwise the organs of pressure sense will become stimulated.

It has been shown (Parts I and II) that while the plastron is mechanically resistant to differences of pressure, the gases within the plastron are rapidly equilibrated to the tension in the external medium. The rate of equilibration may be obtained from the equation

$$\frac{dO}{dt} = Ai_0(t_0 - p_0) - q \quad \text{and} \quad p_0 = \frac{O}{V},$$

where O = oxygen in tracheal system and plastron (assumed at uniform tension), A = area of plastron, i_0 = invasion coefficient of oxygen, t_0 = external oxygen tension of medium, p_0 = internal oxygen tension, q = respiratory rate (c.c. oxygen/sec.), V = total volume of plastron and tracheal system. The solution to this equation is of the exponential form

$$P_0 = K_1 e^{-\lambda t} + K_2,$$

the values of K_1 and K_2 depending on the boundary conditions, but the time constant λ , which is a measure of the rate of equilibration, is given by

$$\lambda = \frac{Ai_0}{V} \text{ secs.}$$

Hence the time required for oxygen in the plastron to fall to $1/e$ of its equilibration value will be

$$\frac{1}{\lambda} = \frac{V}{Ai_0}.$$

Putting $A = 1.0$ sq.cm., $V = 0.9 \times 10^{-3}$ c.c., * $i = 4.8 \times 10^{-4}$,

$$1/\lambda = 0.9 \times 10^{-3} / 1.0 \times 4.8 \times 10^{-4} = 1.9 \text{ sec.}$$

Any change, therefore, in the gas tension of the medium will very rapidly influence the pressure in the tracheal system and cause stimulation of the organ of pressure sense.

* The tracheal volume is found by careful measurement to be 0.4 cu.mm.; the plastron is 5×10^{-4} cm. $\times$ 1.0 sq.cm. = 0.5 cu.mm.

However, there seems to be no doubt that this insect is able to distinguish between oxygen lack and change in external pressure. If it is placed in water saturated with nitrogen the response is identical with that displayed in gas-free water, although the organs of pressure sense will not be appreciably stimulated by the latter treatment as the plastron will exchange oxygen for nitrogen, both at atmospheric tension. Even more conclusive evidence is to be obtained by experiments carried out on operated animals. The removal of the organs of pressure sense does not interfere with their reaction to oxygen deficiency. Hence the response to oxygen lack is not located in the specialized pressure receptors. As a critical test of the ability of the bugs to distinguish an actual pressure increase from loss of gas by the plastron due to unsaturation of the medium, a number of bugs were placed in water deficient in nitrogen but containing 20% saturation in oxygen, while controls were placed in water saturated in oxygen and nitrogen at atmospheric tension. The first group gave persistent 'pressure-increment responses', swimming powerfully to break the surface at intervals.

It is clear, therefore, that while the adult bug can respond independently both to oxygen lack and to increase in pressure, it cannot fully distinguish between the two stimuli, since unsaturation in oxygen must, like unsaturation in nitrogen, stimulate the pressure receptors as demonstrated above. The sense organs which are specific to oxygen want will of course be stimulated at the same time. There is apparently no nervous correlation between the organs of pressure sense and the organs of (presumably) chemical sense, whereby apparent pressure increments due to deficiency in the gas content of the water are disregarded; the reaction to these and to real pressure increments is the same, so that when nitrogen is deficient the animal behaves exactly as it would if it were exposed to water lacking in oxygen or to an increase in external pressure.

It should be emphasized that the dangers of oxygen deficiency are only likely to be met in deep, slow-moving or stationary water, and it is not surprising that the response both to pressure increase and oxygen deficiency is the same, viz. powerful upward swimming.

The possibility of deficiency of some gas other than oxygen, e.g. nitrogen, as in the above experiment, is entirely unnatural, and it is scarcely to be anticipated that *Aphelocheirus* would be adapted to meet this exigency.

The possibility that stimulation of the pressure receptors, caused by changes in the gas tension of the medium, might interfere with their natural efficiency as organs of balance must now be considered. In nature the changes in the oxygen content of the water will not be as sudden or as great as under the very artificial conditions of experiment, especially in flowing streams. But changes in oxygen content which occur, notably in the morning and evening, may be comparable in their effect with the small pressure changes involved in the mechanical orientation of *Aphelocheirus* by these sense organs. It may be in this connexion that the concertina-like air sac, placed close to each pressure receptor, is of value. Small fluctuations in the oxygen tension of the water will cause pressure fluctuations in the tracheal system which would tend to be damped by a small and very compressible sac in this position.

Movements of body and muscle might also cause fluctuations in the pressure of the tracheal system which would require to be damped in a similar manner. The range in which damping can occur is very limited, since resistance to external pressure by the tracheal system is the *sine qua non* of plastron respiration. This organ must be capable of withstanding a high pressure, if necessary, but under ordinary conditions it should be capable, within narrow limits, of taking up small pressure changes of short duration. In this respect it would function very similarly to the hydrostatic organs of *Corethra*.

5. THE ORIENTATION OF *APHELOCHEIRUS* IN ITS ENVIRONMENT

When there is sufficient illumination for the animal to recognize objects, or when it is clinging to the substratum, both nymphs and adults will be capable of maintaining position and appreciating the direction and force of water movements. In the absence of contact with the bottom and even when there are no objects in view, the dorsal light response will give the animal an approximate indication of its relation to the surface and the bed of the stream, and, together with its tendency to negative phototaxy, will guide it back to the substratum—even where the incident light is not vertical. Once in contact with a rough object the thigmotactic response takes control. Thus, in accordance with observations in captivity, *Aphelocheirus* spends the greater part of the time during the day crawling on the bottom over stones, probably in search of small chironomids which appear to be its natural food.

When swimming horizontally away from visible objects, both adults and nymphs will be maintained in the correct position with respect to the vertical by the dorsal light response. The adult, however, has a further check on its position through the paired pressure receptors on the first abdominal segment, and will therefore perhaps be more expert in the very turbulent and swift-flowing parts of the stream.

Under conditions of low illumination in deep parts of the stream, or in the dark, the nymph will be unable to maintain position, but the adult relying on the organs of pressure sense will be capable of controlled swimming and will be able to appreciate its depth, rising towards the surface if the pressure is too great or the water deficient in oxygen. The nymph, on the other hand, does not respond to pressure increase unless this is considerable, and the response is not likely to bring it to the surface. It will respond to conditions of low oxygen tension, however, but in the dark its swimming will be uncontrolled. It is not surprising therefore that while the adults are commonly found in the stony, deeper and more turbulent regions towards mid-stream, there is a tendency for the nymphs which are less active to be found in the more sluggish parts clinging to strands of *Potamogeton pectinatus*.

6. COMPARISON OF THE SPECIALIZED PRESSURE RECEPTORS IN *APHELOCHEIRUS* AND *NEPA*

Very few aquatic insects are heavier than water, and it is only in those which can sink passively to the bottom that organs of pressure sense are important. It is very interesting therefore to find that *Nepa*, a stagnant-water form which is heavier than water, has organs of pressure sense superficially similar to those of *Aphelocheirus*.

Although, in general, the sense organs of *Aphelocheirus* are somewhat similar to those of *Nepa*, they are by no means the same in the two insects. *Nepa* has three pairs of organs which are described by Hamilton (1931) as 'three pairs of oval scars at the side of the third, fourth and fifth abdominal sterna' (Fig. 4). Externally these show as oval membranes apparently perforated by small holes and surrounded by a sclerotized ring covered with fine hairs. A section of these organs in *Nepa* (Fig. 5) shows a rich nerve supply running to sensilla of two main types, the first terminating in a tiny papilla, and the second bearing a large flattened scale, recalling in shape toadstools of the genus *Paxillus* or *Clitocybe*. These sensory papillae of the first type are not exposed to the medium but are enclosed in an air space which is covered by an outer membrane which may stand well away from the sensilla. The outer membrane is made up of the overlapping margins of the second or scale type of sensillum. Baunacke concluded that the organs function as hydrostats and that by their means the animal is enabled to keep itself orientated in the right direction by the pressure of water. The lower organ is, of course, under greater pressure than those nearer the surface. He states that if a normal *Nepa* is blinded so that visual orientation is eliminated and allowed to crawl on a small see-saw submerged beneath the water surface it turns round at once when the inclination is reversed, whereas the insect with all the organs put out of action will walk up and down indiscriminately, giving no response to reversal of the apparatus. Oevermann (1936) has confirmed Baunacke's work but has shown that in addition *Nepa* possesses a general sense of position resident in the appendages and persisting after section of the abdominal nerves. By means of this sense the animal, even with its sense organs destroyed, can still give correct responses on the see-saw provided that its body is given additional weight with a small piece of lead. Weber (1930, p. 108), quotes one, Möller, as stating that *Lethocerus* (Belostomatidae) has a pair of more simple static organs composed of the deeply sunk abdominal spiracles with overlying tactile hairs. He, however, does not give the reference, and it has not yet been possible to trace the original paper. On the face of it there does not appear any reason why a bubble-carrying insect such as the *Lethocerus* should need a special organ for the detection of pressure changes, for one would think that the long sensory hairs among the bubble-holding hairs would suffice.

Baunacke's theory that the lower organs, being under greater pressure than the rest, would provide the animal with a means of appreciating its position relative to the horizontal implies a remarkable sensitivity. Yet apparently not in fact greater than that of the pressure organs of *Aphelocheirus*. The organs in *Nepa* are about 2-3 mm. apart; thus at the most only about 5 mm. separates those on the third sternum from those on the fifth. Suppose, for the sake of argument, that the animal is on an incline of 45° ; this will mean that the uppermost sense organ is 1.5 mm. above the lowest. This involves a pressure difference of $0.00015 \text{ atm.} = 0.114 \text{ mm. of mercury}$.

For Baunacke's theory of the mode of action of the sense organs to be plausible two conditions must obtain. First, all parts of the tracheal system must be effectively protected against the entry of water even under pressure. This condition certainly

appears to be fulfilled. Secondly, the gas-containing cavity of the sense organ must be in communication with its fellows via the adjacent spiracles and the tracheal system. This point has hitherto seemed uncertain; though stated by Baunacke to

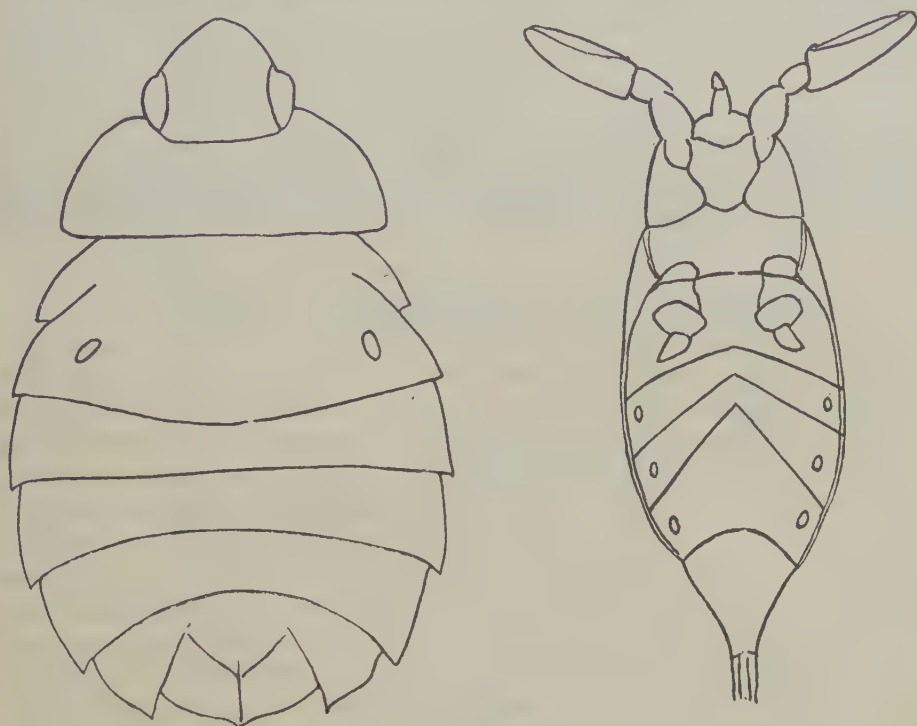


Fig. 4. Diagrammatic ventral view of *Aphelocheirus* and *Nepa* to show position of specific organs of pressure sense.

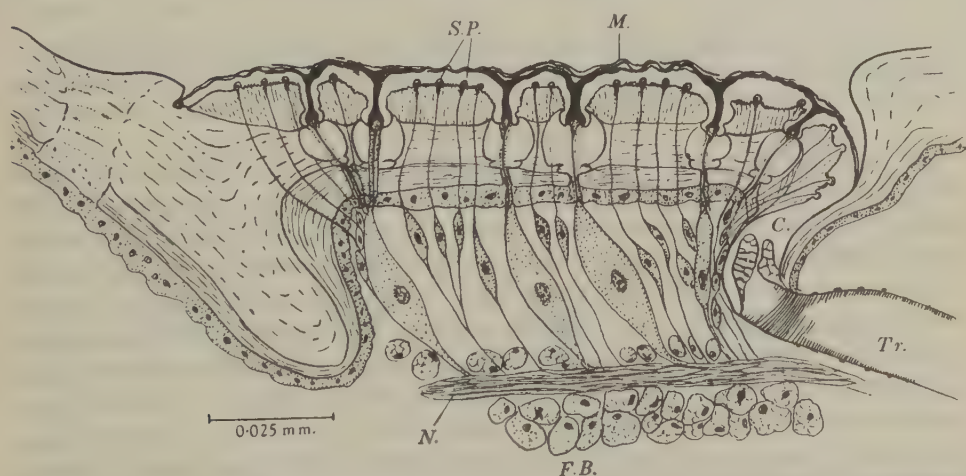


Fig. 5. Section of specific organ of pressure sense of *Nepa*. Tr. = trachea; N. = nerve; M. = membrane composed of overlapping expanded margins of scale sensilla; C. = closed spiracle; F.B. = fat-body cells; S.P. = sensory papillae.

be the case and implied in his argument, it is not clearly evident from his figures and is directly contrary to the observations of Hamilton, who states categorically that the membrane does not cover the site of the spiracle, which lies close beside the organ 'but entirely separate from it'. In view of this contradiction we have made a careful study of the sense organ of *Nepa* by sections, injection and otherwise. As a result we have come to the conclusion that while even excellent sections often appear to support Hamilton's interpretation they are apt to be misleading where the exact point of attachment of such a thin membrane is concerned, and we are convinced that Baunacke's interpretation is essentially the correct one. This being so the organs of *Nepa* must be considered as constituting a single system connected by the trachea.

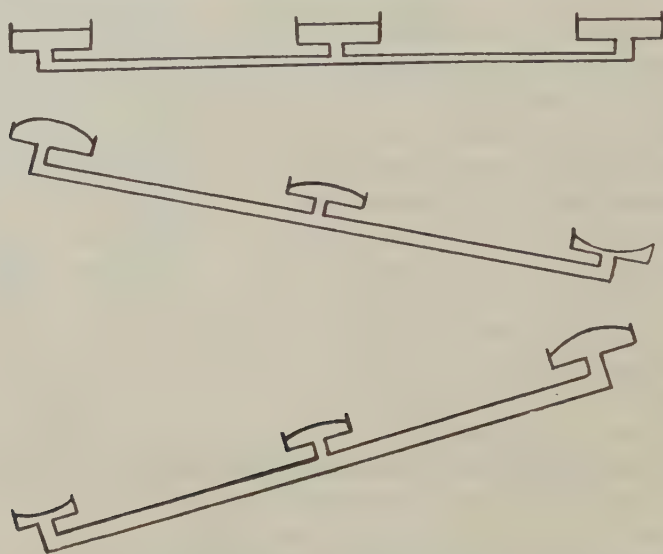


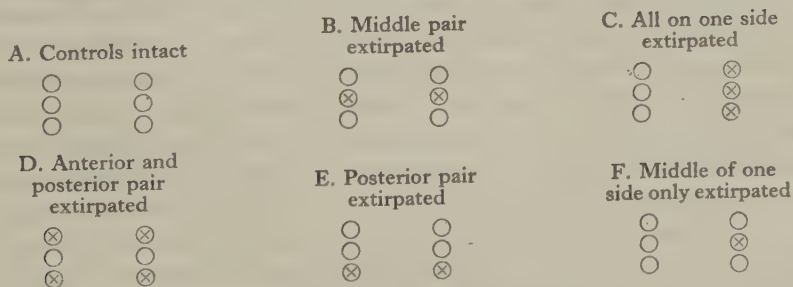
Fig. 6. Diagram to show the principle of action, when tilted in water, of a system composed of three distensible membranes enclosing an air space and connected by an air-filled tube, comparable to the three pressure receptors of one side in *Nepa*.

Since the membranes, particularly because of their laminated structure, must have considerable elastic properties, and since the sensilla of the first type are short, vertical and rigid, we must look upon a single organ not as being sensitive to a wide range of pressures but as constructed primarily to register whether the force acting on the membrane is tending to pull it out or push it in and whether or not the membrane is touching the small sensory papillae. Thus the system of organs must be regarded as acting according to the scheme shown in Fig. 6, the membrane of the uppermost organ being convex and that of the lowest concave. Such a system would respond with great sensitivity to *relative* changes as between different organs in the series, but not necessarily to changes of pressure affecting the whole animal uniformly.

Since *Nepa* is a soft-bodied insect, and its tracheal system does not require to be highly resistant to external pressure, the air in the tracheal system will be effectively

exposed to the external pressure of the atmosphere and the head of water above it. Hence the pressure in the tracheal system which is applied at the internal surface of the membranes will always approximate to the pressure outside the membranes, thus allowing a sensitive all-or-none response to differences of pressure due to the animal's position relative to the vertical. *Nepa* is unlikely to be troubled by eddy-pressure effects as it lives in still water.

We have carried out a number of experiments in which intact *Nepa* were exposed to increases of pressure up to 1 atm., but in no case have we been able to detect any response. This goes some way to confirming the above conclusion as to the mode of action of the sense organs. Moreover, it must be remembered that *Nepa* is very sluggish, is incapable of vigorous swimming, and is essentially a shallow-water animal dependent for its oxygen supply to a small extent on the oxygen content of the water, but mainly on the atmosphere. In the former method it raises the wing cases and exposes a shallow groove into which open the spiracles, and over which long hydrofuge hairs are extended to form a roof; in the latter method the posterior siphon is pushed out of the water, after gaining access to the surface by climbing up a plant or other object. As long as it can maintain its orientation in the vertical axis absolute pressure changes, within the limits it is likely to encounter, are probably immaterial to it. We have repeated Baunacke's experiments using a somewhat smaller apparatus in which the movable plank of the see-saw (20×4.3 cm.) is enclosed in a glass tube 4.8 cm. in diameter to prevent the animal from falling off the sides. The tube is closed at each end by a perforated cork, and the whole, filled with water, immersed in a tank of water 30 cm. deep. Instead of blinding our *Nepa* we carried out the experiments in the dark room, the tank being illuminated from one side by a very dim and diffuse Wratten Safelight no. 3. Under these conditions visual orientation seemed to be eliminated, and we were able satisfactorily to confirm Baunacke's results. Both Baunacke and Oevermann, however, had been content only to investigate the effect of eliminating *all* the pressure receptors, whereas we, in order to secure more information about the mode of action of the system, wished to study the consequences of extirpating some and leaving others intact. Accordingly, we divided a collection of thirty bugs into six lots and extirpated certain of their pressure sense organs according to the following scheme:



These groups were then tested on the see-saw under uniform conditions in the dark room and the number of correct turns immediately following a tipping of the see-saw was recorded in order to obtain a sample estimate of the probability P of a

successful response for each bug. It was found that the control lot A gave a total of 68 'correct' responses in 92 tests, the individual values of P being 0.69, 0.94, 0.835, 0.72, 0.61 and 0.5, giving a mean value of $\bar{P}=0.718$. Lot B was not significantly different, 43/56 and $\bar{P}=0.718$. Lots C and D were so seriously affected that the numerical significance of the tests was doubtful; but while two individuals in lot C gave occasional positive results (7 in 40) all those in D were negative. In general, all the insects in these lots were clumsy, sluggish and disorientated, lot C, in particular, being apt to turn aimlessly in circles while lot D showed in fact just as complete loss of power to respond to tilting of the see-saw as do insects with all the sense organs eliminated. Lot E were rather sluggish and slow so that experiments were difficult to perform, but, nevertheless, ability to respond was surprisingly little impaired, figures being 27/38, $\bar{P}=0.544$. Finally, lot F, in which activity was greater than in E, gave results—49/115, $\bar{P}=0.413$ —showing that the power to respond, though still present, was at a yet lower level. The significance of the results was determined as follows. The total variance for each group of individuals referred to as 'lot' was derived from the sum of the squared deviation from the mean due to the individual differences of each animal and of the calculated random variance due to sampling for each set of trials. The t test was then carried out between the various pairs of lots A, B, C, etc., using this total variance. Using this test it was found that the difference between A and B is negligible, and the effect of operation E is also outside the level of significance ($P=0.2$). The difference between F and the control is, however, fully significant ($P=0.01$), as are also, of course, the quite overwhelming effects on lots C and D.

The results as a whole indicate that for the response to persist it is necessary to have at least two of the organs symmetrically intact on each side. They also suggest, though they do not prove, that the anterior and posterior pair together make a slightly more efficient system than any two pairs of adjacent organs, and they show quite conclusively that the destruction of one organ only is more disorientating than the destruction of two symmetrically. All these conclusions seem to give satisfactory further support to the theory of action put forward by Baunacke—though it is a little surprising that elimination of the middle pair has generally no observable effect whatever. Thus the principle of action of the pressure-organ system of *Nepa* is essentially comparative, the relative pressure differences at each end of the system being registered by distensible membranes. Finally, there is a curious and as yet unexplained result of extirpation of the abdominal sense organs which seems worth putting on record. The operated and the control stocks of *Nepa* were kept in large shallow dishes of water, each containing a stone which projected above the surface. From time to time the operated individuals would emerge from the water and stand motionless on the stone for hours at a stretch, their front and middle legs flexed and their hind legs usually fully extended so as to raise the 'siphon' and hinder part of the body to its fullest extent, the inclination of the body often being more than 45° so that the insect appeared to be standing on its head. A remarkable feature of the performance was the tolerance to desiccation when in this state. Normally, *Nepa* are very sensitive to drying, and 10 min. or so in a dry atmosphere is usually enough to break the stubborn cataleptic state which is such a well-known characteristic of

this animal's behaviour (see Brocher, 1916; Steiniger, 1936). This abnormal stance and tolerance to desiccation were observed occasionally in all the groups B-F inclusive, but never in the controls. The periods in between the acrobatic bouts are evidently considerable, for at any one time there were usually one or two insects out of the 25 in this state but seldom more than three or four.

We have already noted the fact that it is vital for *Aphelocheirus*, in view of the risks to which it is exposed, to have some type of sense organ which will be rapidly sensitive to a wide range of pressure changes affecting the whole animal as it ascends or descends. For this animal therefore the type of organ found in *Nepa* would be unsuitable, since it appears to give an all-or-none response rather than a finely graduated one, and in *Aphelocheirus*, whose tracheal system is maintained at a pressure independent of the surroundings, it would function only at one level. Indeed, the mode of action of the plastron precludes, and its structure is designed to prevent, the equalization of pressure between the tracheal system and surrounding water. The type of organ actually found in *Aphelocheirus* is capable of graded stimulation as the hairs are depressed and the sensory end-organs successively touched. It would be unnecessary for *Aphelocheirus* to possess more than one pair of such organs, since it very rapidly traverses a distance equivalent to its own length.

It has been shown that the two sense organs of *Aphelocheirus*, interconnected as they are via the tracheal system, do function as a 'differential manometer' enabling the insect to keep on an even keel in the transverse plane while swimming rapidly forwards, as well as giving the animal some absolute pressure sense. The function of balance might be achieved as a result of the comparison of sensory input from the two organs were their cavities not in communication; but it seems clear that such an arrangement would be inefficient compared to the existing one.

SUMMARY

1. *Aphelocheirus* lives in swiftly flowing streams, an environment in which, chiefly because of its method of plastron respiration, it must be able to maintain itself in a particular stretch of river and must especially have some safeguard against sudden and uncontrolled descent into regions of low oxygen tension. These necessary powers of orientation are provided: (a) by the ordinary type of tactile trichoid sensillum, (b) by light-sensitive organs, and (c) by specialized pressure receptors.

2. When stationary or when climbing on weeds and stones the insect should have no difficulty in appreciating direction and rate of flow of the water by means of its antennae and trichoid sensilla. When swimming the dorsal light response, light compass orientation and probably also rudimentary form sight enable the animal to maintain position except when in deep smoothly flowing water under conditions of very low illumination.

3. A certain gross sensitivity to pressure changes probably resides in the proprioceptors of the cuticle, but, in addition, there are specialized pressure receptors which consist of a pair of oval depressions on the sterna of the second abdominal segment closely associated with the spiracles of that segment and bearing a pile of long recumbent hydrofuge hairs, approximately 60,000 per sq.mm., holding a film of gas.

Special tactile sensilla are interspersed among the hydrofuge hairs. The hairs move with the movements of the air-water interface which result from changes in pressure. Experiments have shown that these two organs are exceedingly sensitive both to uniform and differential pressure changes, and that by their means the animal is enabled to direct its swimming upward or downward and also to maintain an even keel irrespective of directional light stimulation.

4. The adult bug responds immediately by jerky upward swims when exposed to sudden increase of pressure, and the same response is also elicited independently by oxygen lack. But the insect cannot fully distinguish between the two stimuli and is unable to distinguish loss of gas by the plastron due to unsaturation of the medium from an actual pressure increase. Thus bugs placed in water deficient in nitrogen but containing 20% saturation in oxygen give persistent pressure-increment responses.

5. The possibility is considered that stimulation of the pressure receptors caused by changes in the gas pressure in the tracheal system, due to muscle or body movements or to changes in the gas tension of the medium, might interfere with their efficiency as organs of balance. It is suggested that the small, very compressible, concertina-like air sacs associated with each sense organ serve to damp out such fluctuations and so avoid this difficulty.

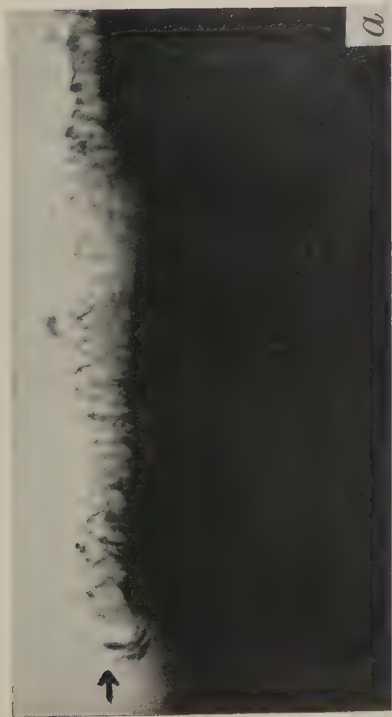
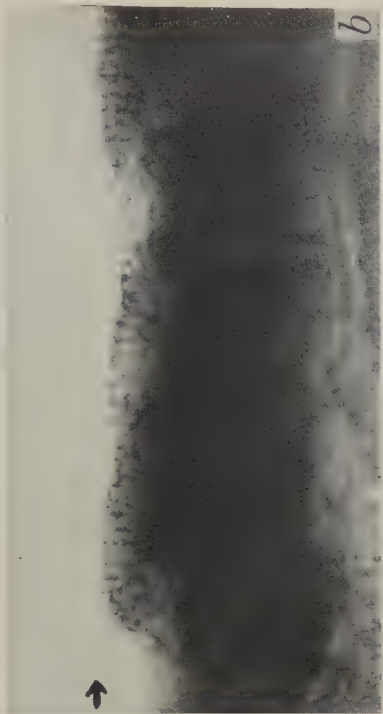
6. *Aphelocheirus* nymphs lack the specific pressure receptors. They are accordingly more dependent on directional light for orientation, and in the dark behave as do adults with the pressure receptors extirpated.

7. Aquatic insects which carry substantial air bubbles with them and so are lighter than water have a ready means of perceiving pressure changes by volume changes of the bubble. *Aphelocheirus* being heavier than water and carrying no bubble therefore needs the special pressure receptors. Similarly, *Nepa*, which is also heavier than water, has specialized pressure receptors. The organs of the latter are redescribed and their mode of action compared with those of *Aphelocheirus*. The action of the *Nepa* organs is not finely graduated as in *Aphelocheirus* but is of the 'all-or-none' type. The three pairs of organs in *Nepa* are co-ordinated into a single differential manometer system giving no response to changes in absolute pressure but conferring high sensitivity to relative changes as between different parts of the body and correspondingly accurate orientation in the vertical axis. It is shown how the two types of sense organ are exactly suited to the peculiar contingencies to which the insects are exposed by their particular environments. The experiments of Baunacke on *Nepa* are repeated and extended and his conclusions confirmed.

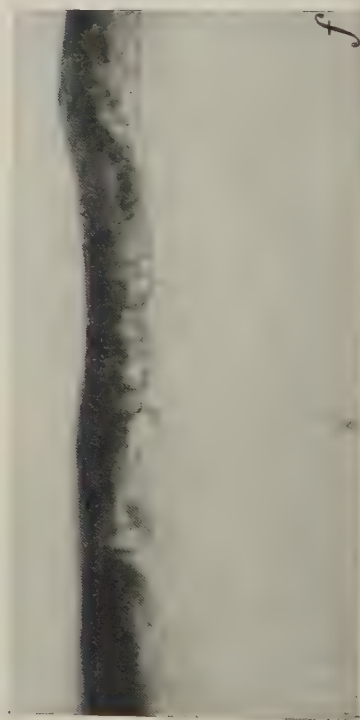
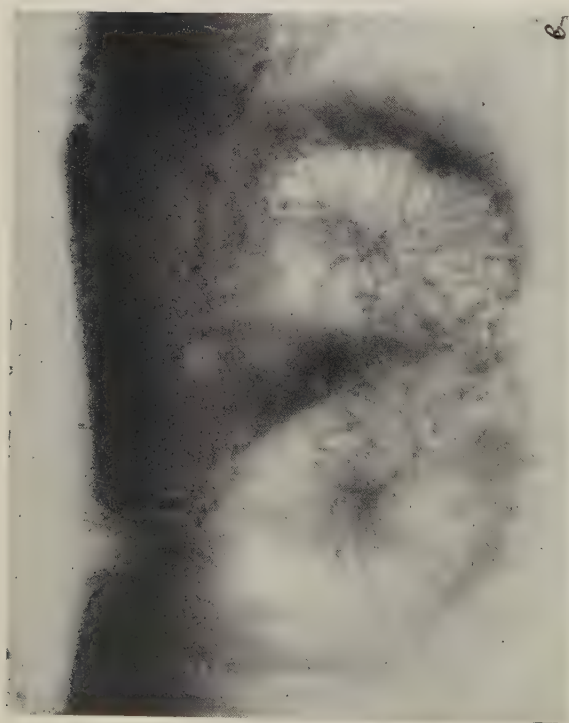
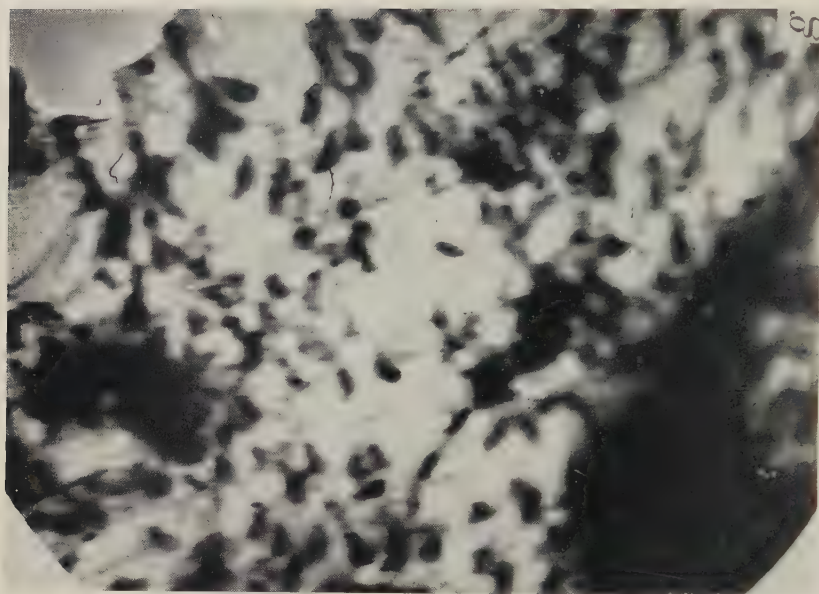
We are very grateful to Mr W. E. China for his efforts (unfortunately unsuccessful) to trace the Möller reference for us.

REFERENCES

- BAUNACKE, W. (1912). *Zool. Jb. (Abt. Anat.)*, **34**, 179.
 BROCHER, F. (1916). *Arch. Zool. exp. gén.* **55**, 483.
 HAMILTON, M. A. (1931). *Proc. Zool. Soc. Lond.* p. 1067.
 OEVERMANN, H. (1936). *Zeit. wiss. Zool.* **147**, 595.
 STEINIGER, F. (1936). *Ergbn. Biol.* **13**, 348.
 THORPE, W. H. & CRISP, D. J. (1947). Parts I and II. *J. Exp. Biol.* **24**, 227, 270.
 WEBER, H. (1930). *Biologie der Hemipteren*. Berlin.



THORPE AND CRISP—STUDIES ON PLASTRON RESPIRATION



THORPE AND CRISP—STUDIES ON PLASTRON RESPIRATION

ON THE FUNCTION OF HAEMOGLOBIN IN *CHIRONOMUS* AFTER OXYGEN LACK

By BARBARA M. WALSHE
Bedford College, University of London

(Received 17 March 1947)

(With Four Text-figures)

INTRODUCTION

The function of haemoglobin in the large chironomid larvae of the *Chironomus plumosus* group has been studied experimentally by Ewer (1942). She compared the oxygen consumption of normal larvae at different pressures of oxygen with that of larvae whose respiratory pigment had been rendered functionless by treatment with carbon monoxide, and from the differences between the two deduced the extent to which the haemoglobin is of functional value to the animal in the transport of oxygen. Her results show that at 17° C. the haemoglobin of *Chironomus* larvae (previously kept in aerated water) does not function at air saturation of the water, but only at oxygen concentrations below 3 ml./l. (44% saturation). This can be correlated with the common occurrence of *Chironomus* larvae in stagnant habitats known to be more or less depleted of oxygen from time to time.

The work of Ewer in part repeats, under careful experimental conditions, similar work done by Harnisch (1936), whose experimental technique is open to criticism. Harnisch studied both animals previously kept in oxygen gas and animals which had been subjected to severe oxygen lack by keeping them about 20 hr. in nitrogen gas before experiments. The nitrogen-treated larvae when replaced in aerated water in a Barcroft manometer had an oxygen uptake 160% that of the oxygen-treated larvae. This indication of the repayment of an oxygen debt persisted for about 3 hr. The increased oxygen uptake of the nitrogen-treated animals was observed not only in aerated water but also at lower oxygen pressures, even at 14% air saturation. The oxygen consumption of nitrogen-treated larvae which had also been subjected to carbon monoxide was, however, lower than that of nitrogen-treated larvae whose haemoglobin had not been converted into carboxyhaemoglobin; this was so in water at all oxygen pressures at and below air saturation. Harnisch concluded that when the metabolic rate rises in recovering from oxygen lack the haemoglobin becomes functional in oxygen transport at all pressures of oxygen, and that the increased oxygen consumption of recovering larvae is, thanks to their haemoglobin, maintained irrespective of the oxygen content of the water. That the larvae, which must frequently experience anaerobic conditions in nature, should thus be able to recover from oxygen lack even in water containing very little oxygen, Harnisch considers to be of paramount importance in assessing the function of the haemoglobin. This

work is, however, open to criticisms, some of which have been made by Ewer (1942). The temperature varied from 16 to 23° C. in experiments supposed to be comparable and Harnisch used carbon monoxide pressures high enough to have had an inhibitory effect on cellular oxidations. He dismisses this possibility with the statement that although Warburg (1926) had shown such a concentration to have an inhibitory effect on yeast he himself considers that 'bei der Hefe der Kontakt zwischen dem Giftgas und den Zellen doch wohl wesentlich inniger war als bei meinen Tieren'! He applies no statistical tests to his data, and two series of estimations of the metabolism in aerated water of animals after nitrogen treatment (Table 2, p. 396 and Table 6, p. 406) give significantly different averages.

For these reasons a further investigation of the metabolism of chironomid larvae after oxygen lack was considered necessary.

MATERIAL AND METHODS

The chironomids used were the final instar larvae of *Chironomus plumosus* L. collected from the mud of Regent's Park Lake, London. The species was identified by means of Edwards's key (1929) from adults which emerged in the laboratory, and checked by comparison with the collection at the British Museum. I wish to thank Dr J. Smart for allowing me access to this collection. Larvae were collected 1 or 2 days before experiments and were kept in the laboratory in aerated water in shallow dishes with a thin layer of mud, under which conditions they remained healthy, and successfully pupated and emerged.

The respiration of larvae was measured in an apparatus in which the larvae were subjected to a continual slow stream of water, their oxygen consumption being calculated from the difference in oxygen content of the water before and after passing through the respiratory chamber. A diagram of the apparatus is given in Fig. 1. A 5 l. aspirator (*a*), containing aerated buffered water (0.004 *N* solution of NaHCO₃) was connected by way of a water-mixer (*b*), to the respiratory chamber (*c*). This consists of an 11 cm. length of wide glass tubing, 1.5 cm. diam., blackened on the outside except for a longitudinal unpainted slit about 5 mm. wide through which the behaviour of the larvae could be observed without exposing them to excessive light, which has an activating effect. Perforated porcelain disks on the rubber bungs prevented the larvae from crawling out of the respiratory chamber. The three-way tap (*f*) and capillary outflow tube (*d*) allowed samples of the water flowing into and out of the respiratory chamber being taken for analysis of dissolved oxygen. This was determined by the syringe-pipette micro-Winkler method described by Fox & Wingfield (1938). The nozzle of the syringe-pipette was inserted into the rubber tubing at (*f*) or (*d*) and water samples of 1.4 ml. thus removed. The rate of flow through the apparatus was kept steady by maintaining a constant level in the aspirator (*a*) by means of a continuous inflow from flask (*g*) and a simple overflow device, and by firmly clamping the whole system of tubing to prevent any shift in position. The rate was determined by the length of time taken by the outflowing drops to fill a given volume. The respiratory chamber, together with the inflow and outflow tubes, were supported in a large thermostatic water bath.

Between thirty and forty larvae were used in each experiment. With a water flow of 1.3 ml./min. these larvae lowered the oxygen content of the water by about 20% at 17° C. From the difference in oxygen content of the water before and after passing through the respiratory chamber and the rate of flow of the water, the oxygen consumption of the larvae was calculated in cu.mm. oxygen/g. (wet weight)/hr.

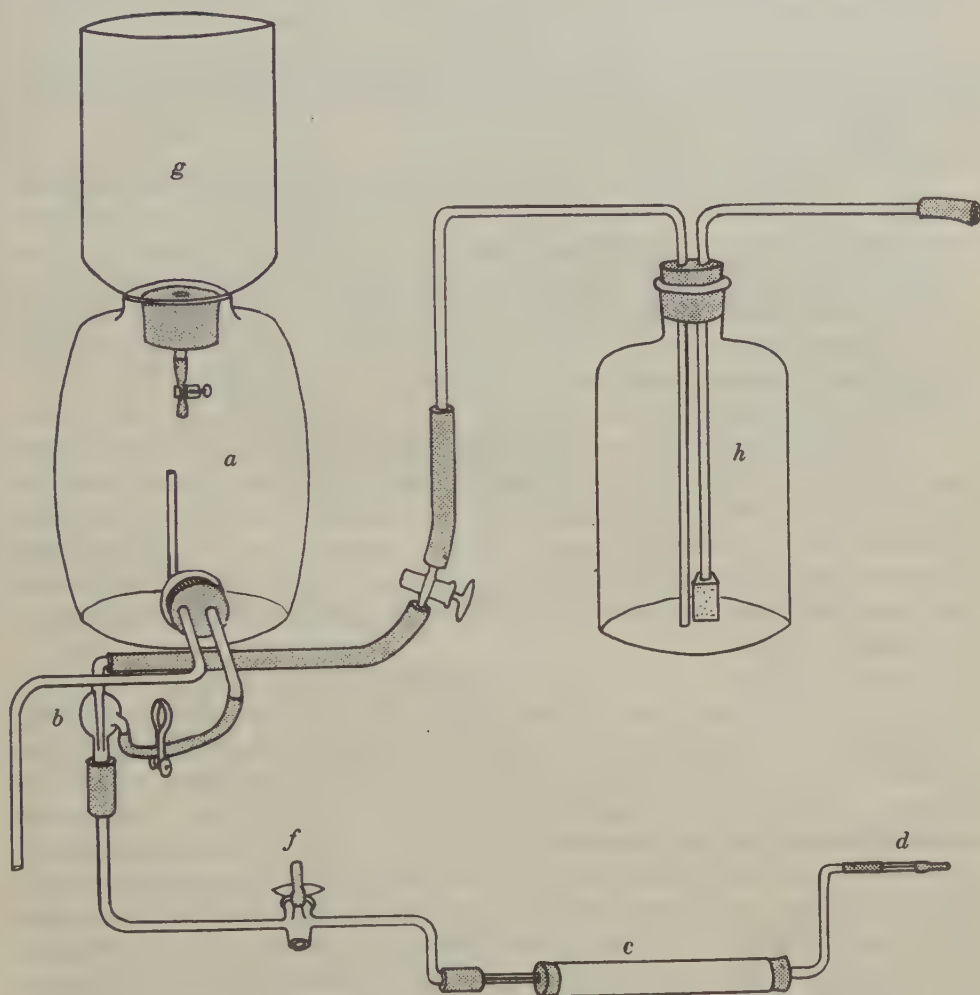


Fig. 1. Apparatus for measuring the oxygen consumption of *Chironomus* larvae.
For explanation see text.

The 3 l. bottle (*h*) contained nitrogenated water (oxygen content less than 0.1 ml./l.); this could be driven through the respiratory chamber by the pressure of gas from a nitrogen cylinder, the larvae being in this way subjected to a period of oxygen lack.

Experiments were of about 24 hr. duration. Larvae were put into the respiratory chamber in flowing aerated water and left for about 2 hr. in order to reach a steady

state of activity. The oxygen contents of inflowing and outflowing waters were then determined at intervals of approximately 45 min. for the next 3 hr., and from these determinations the average metabolic rate of the animals was determined. The inflow of aerated water was then stopped and nitrogenated water slowly forced over the animals. The larvae were left thus for 16 hr., at the end of which period the nitrogenated water supply was cut off and aerated water once again allowed to flow through the apparatus. Oxygen samples of the outflowing water were taken at intervals of 5 min. for the next hour and at frequent intervals (generally 15 min.) after that until the oxygen uptake became constant and equal to that found before the anaerobic period. A complete record of the metabolism of larvae recovering from anaerobiosis was thus obtained.

The rate of replacement of oxygen-free water present in the respiratory chamber by inflowing aerated water was determined by taking frequent oxygen samples of the outflowing water with no animals present in the chamber. The mixing was slow, nearly an hour elapsing before the water in the chamber was completely replaced by fully aerated water. Larvae therefore commenced recovery from anaerobiosis in water of low oxygen content and were subsequently subjected to progressively higher oxygen concentrations until air saturation was finally reached.

Most experiments were carried out at 17° C. One series, however, was made at 1–2° C. This low temperature was obtained by surrounding the respiratory chamber and tubes with crushed ice. The purpose of these experiments was to reduce the activity of the animals while estimations of the metabolic rate were being made: the larvae spent the overnight anaerobic period, however, at 17° C. and were thus allowed to accumulate as much oxygen debt, if any, as those in previous experiments. The larvae were again chilled at the end of the overnight period before the aerated water was turned on.

A number of experiments were made on the recovery from oxygen lack of larvae whose haemoglobin had been rendered functionless by conversion to carboxy-haemoglobin. The larvae were kept in darkness for 3 hr. previous to experiments in aerated water containing sufficient carbon monoxide-saturated water to make the pressure of dissolved carbon monoxide one-sixth that of the oxygen. This concentration of carbon monoxide was sufficient to convert the haemoglobin entirely into the carboxy-compound, but at this low concentration it was very unlikely to have had any inhibitory effect on cellular oxidations, for which, owing to the low affinity of oxidases for carbon monoxide, relative pressures considerably greater than one are necessary (Keilin, 1929; Wolsky, 1938). Experiments with larvae so treated were carried out in the same manner as those with normal larvae, except that the water contained 0.2 ml./l. carbon monoxide to prevent dissociation of the carboxy-haemoglobin. The small amount of light introduced through the slit in the respiratory chamber did not cause any dissociation. Individual experiments with carbon monoxide-treated animals were alternated with control experiments with normal animals. This was a necessary precaution to ensure that larvae in the same physiological state were being compared, since seasonal differences in metabolic rate were found to occur.

At the end of all experiments the larvae were dried on filter paper and weighed. In the carbon monoxide experiments a drop of the animal's blood was then examined with a hand spectroscope: the absence of fading of the absorption bands after the addition of sodium hydrosulphite indicated that no dissociation of carboxy-haemoglobin had occurred during the experiment.

RESULTS

The results of a series of seven experiments on normal animals at 17° C. are given in Table 1. Fig. 2 shows graphically the changes in metabolic rate during the course of the experiments. The oxygen uptake of an average basal value of $191.7 \pm 7.8^*$ cu.mm./g. (wet weight)/hr. before nitrogen subjection rose within 5 min. of the introduction of aerated water to a significantly higher value, the

Table 1. *Oxygen consumption of larvae of Chironomus plumosus L. at 17° C. before and after 16 hr. in nitrogenated water. Normal animals, May-June*

Time before anaerobiosis	Oxygen concentration ml./l.	Oxygen consumption, cu.mm./g. (wet weight)/hr.		
		Separate values	Mean	Grand mean and S.E.
4-3 hr.	6.5	293, 158, 179, 163, 188, 184	194	191.7 ± 7.8
3-2	6.6	209, 150, 164, 168, 274, 198	194	
2-1	6.6	197, 166, 163, 179, 243, 187, 252, 163	194	
1-0	6.6	155, 148, 156, 176, 158, 204, 262, 280, 173, 156	187	
Time after anaerobiosis				
0-5 min.	0.4	12, 16, 33	20	235.0 ± 13.8
5½-10	4.2	284, 227, 147, 288, 298, 231, 307	255	
10½-15	5.2	155, 210, 210, 230, 224, 243	212	
15½-20	5.8	172, 137, 117, 243, 235, 198	184	
20½-25	6.2	204, 129, 242, 237, 280, 185	213	204.5 ± 9.3
25½-30	6.5	267, 108, 212, 213, 161, 249, 228	205	
30½-40	6.5	98, 187, 271, 217, 208, 243	204	
40½-60	6.4	143, 238, 236, 217, 260	219	
1-1½ hr.	6.5	84, 224, 216, 235	190	188.4
1½-2	6.4	22, 210, 217, 273, 215	187	

average metabolic rate 5-15 min. afterwards being 235.0 ± 13.8 . This marked increase only lasted for about 10 min., subsequent values from 20 min. to 1 hr. after the end of anaerobiosis being only slightly greater than the basal rate of the previous day. The statistical significance of this slight increase is very doubtful: the averages are 204.5 ± 9.3 after, as compared with 191.7 ± 7.8 before, anaerobiosis, with the large probability value (*p*) of 0.27. Further, an analysis of variance of these data did not indicate a significant increase in metabolic rate. At the end of 1 hr. the oxygen consumption had returned to the basal rate.

The initial rise in oxygen consumption was accompanied by great activity on the part of the animals: the larvae, which had been completely inactive in nitrogenated water, started violent undulatory movements of the body upon the introduction of aerated water. It seemed likely that the increased oxygen uptake was due to this

* Standard error.

activity rather than to the repayment of an oxygen debt; to test this experiments were made in which the activity changes were eliminated by chilling the animals. The results of six such experiments are given in Table 2 and Fig. 3. The basal metabolic rate of such larvae was 25.0 ± 2.4 cu.mm./g./hr. After the 16 hr. anaerobic period the metabolism rose slowly during the first 25 min. to a value about 160% of the basal rate and maintained this high level for at least 2 hr. The difference in metabolic rate before and 10 min. to 2 hr. after the anaerobic period is highly significant.

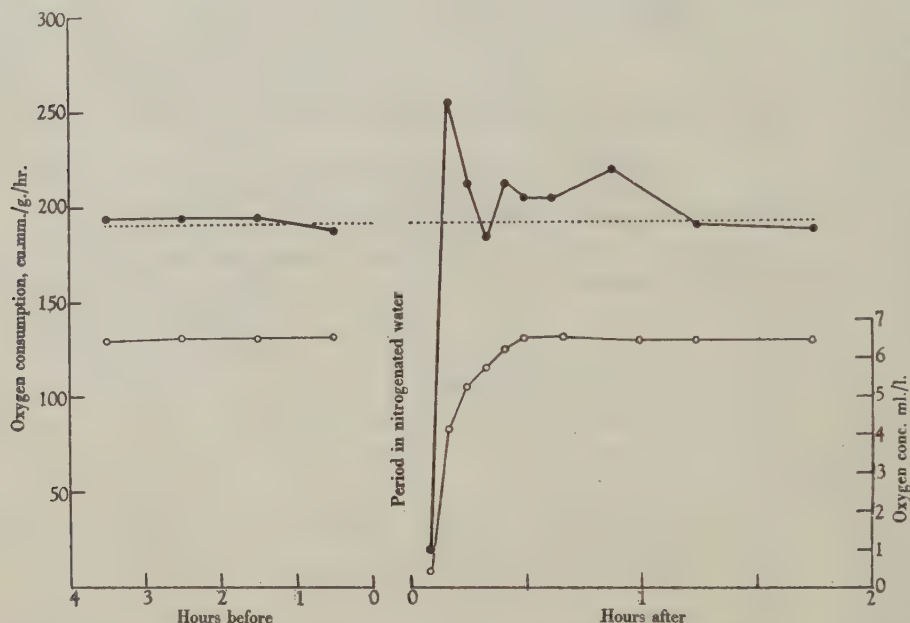


Fig. 2. Rates of oxygen consumption at 17°C . of *Chironomus plumosus* larvae before and after a 16 hr. period of anaerobiosis. Normal animals, May-June. ●, oxygen consumption; ○, oxygen concentration in respiratory vessel. The broken line represents the average metabolism before nitrogen subjection. Data from Table 1.

In interpreting the results at 17°C ., therefore, the initial increase in oxygen consumption must be attributed to the great increase in activity of the larvae at that time, since immobile, chilled larvae do not show it. Larvae at both temperatures, however, showed a subsequent more prolonged increase in metabolism, and although at 17°C . the increase is so slight that its reality is doubtful, at the lower temperature it is greater.

The results of a third series of experiments in which the recovery from anaerobiosis of normal animals was compared with that of carbon monoxide-treated larvae are given in Tables 3 and 4 and Fig. 4. The metabolism curve is of the same general form as that already described for normal larvae at 17°C . except that the basal level is lower, being 140.3 ± 4.0 cu.mm./g./hr. compared with 191.7 ± 7.8 cu.mm./g./hr. previously. This must be due to a seasonal change, these last series of experiments having been made in late September on animals which would have

over-wintered as larvae, while the first series was made in May and June on larvae of a summer generation.

The basal metabolic rate of larvae with carboxyhaemoglobin is seen to be the same as that of untreated larvae. This confirms Ewer's conclusion that in aerated

Table 2. Oxygen consumption of larvae of *Chironomus plumosus* L. at 1° C. before and after 16 hr. in nitrogenated water. Normal animals, July

Time before anaerobiosis	Oxygen concentration ml./l.	Oxygen consumption, cu.mm./g. (wet weight)/hr.		
		Separate values	Mean	Grand mean and s.e.
2-1½ hr.	5.7	35.3, 16.8, 16.0, 18.9, 26.9	22.8	25.0 ± 2.4
1½-1	5.8	52.2, 20.9, 7.3, 24.2, 27.9	26.5	
1-1½	5.8	36.7, 33.6, 19.5, 8.6, 20.6, 21.6	23.4	
½-0	5.8	31.3, 39.3, 19.5, 22.8	28.2	
Time after anaerobiosis				
0-5 min.	0.3	0, 19.3, 9.8, 0, 9.4, 0	6.4	38.9 ± 2.1
5½-10	1.7	42.2, 48.2, 0, 0, 17.7	21.6	
10½-15	3.6	16.3, 15.7, 18.1, 19.6, 87.9, 38.7	32.7	
15½-20	4.6	29.2, 14.3, 44.7, 40.0, 49.4, 39.2	36.1	
20½-25	5.1	6.8, 26.4, 53.0, 52.1, 56.2, 37.8	38.7	
25½-30	5.4	14.3, 45.0, 56.5, 57.0, 52.8, 37.2	43.8	
30½-40	5.6	14.3, 47.2, 41.9, 55.8, 54.6, 25.6, 22.2, 48.7, 46.1, 51.6, 61.3, 37.2	42.2	
40½-60	5.7	29.9, 48.7, 46.8, 46.2, 42.8, 43.3, 41.2, 57.6, 26.9, 41.9, 41.2, 33.3, 52.2, 41.2, 37.6	43.4	
1-1½ hr.	5.9	44.7, 33.4, 46.8, 31.4	39.1	34.5
1½-2	5.8	51.4, 30.0, 29.3, 22.8, 39.2	34.5	

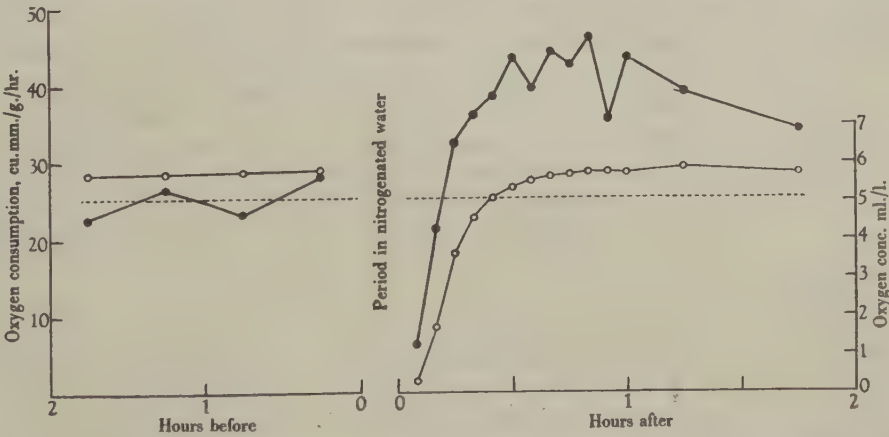


Fig. 3. Rates of oxygen consumption at 1° C. of *Chironomus plumosus* larvae before and after a 16 hr. period of anaerobiosis. Normal animals, July. ●, oxygen consumption; ○, oxygen concentration in respiratory vessel. The broken line represents the average metabolism before nitrogen subjection. Data from Table 2.

water the haemoglobin is not functional, and shows that treatment with carbon monoxide had not affected cell oxidations in the larvae. The metabolism of the treated larvae after the anaerobic period, on the other hand, differed from that of normal larvae. The initial increase in oxygen consumption caused by activity did not occur, although the activity of the treated larvae appeared in no way diminished.

The larvae show this increased activity at a time when, owing to the slow rate of flow through the respiratory chamber, the inflowing aerated water has only partly replaced the nitrogenated water, the oxygen concentration inside the chamber

Table 3. *Oxygen consumption of larvae of Chironomus plumosus L. at 17° C. before and after 16 hr. in nitrogenated water. Normal animals, September*

Time before anaerobiosis	Oxygen concentration ml./l.	Oxygen consumption, cu.mm./g. (wet weight)/hr.		
		Separate values	Mean	Grand mean and s.e.
3-2 hr.	6.8	146, 160, 114, 176	149	140.3 ± 4.0
2-1	6.7	143, 162, 148, 156, 95, 121	138	
1-½	6.7	128, 145, 146, 136, 156, 109	137	
½-0	6.7	117, 128, 170, 123, 142, 145, 151, 149	141	
Time after anaerobiosis				
0-5 min.	1.0	34, 28, 76, 56	48	189.5 ± 14.0
5½-10	4.3	238, 177, 231, 238, 205	218	
10½-15	5.1	165, 122, 208, 195, 115	161	
15½-20	5.7	88, 106, 226, 196, 112	145	
20½-25	6.0	109, 108, 150, 139, 174	136	141.6 ± 4.0
25½-30	6.3	128, 103, 164, 148, 121	133	
30½-35	6.3	98, 97, 128, 154, 137	123	
35½-40	6.5	128, 147, 135, 145, 125	136	
40½-50	6.6	130, 121, 176, 142, 152	144	
50½-60	6.6	146, 126, 198, 153, 136	152	
1-1½ hr.	6.7	166, 138, 170, 174, 156	161	
1½-2	6.7	154, 142	148	

Table 4. *Oxygen consumption of larvae of Chironomus plumosus L. at 17° C. before and after 16 hr. in nitrogenated water. Carbon monoxide-treated animals, September*

Time before anaerobiosis	Oxygen concentration ml./l.	Oxygen consumption, cu.mm./g. (wet weight)/hr.		
		Separate values	Mean	Grand mean and s.e.
3-2 hr.	6.4	145, 138	142	143.3 ± 5.2
2-1	6.5	184, 107, 150, 119, 119	136	
1-½	6.4	154, 135, 131, 142, 126, 158	143	
½-0	6.4	187, 149, 162, 102, 126, 163, 158	149	
Time after anaerobiosis				
0-5 min.	0.7	21, 26, 30, 9	21	129.0 ± 9.6
5½-10	4.0	103, 137, 124, 75, 141	116	
10½-15	5.1	128, 168, 121, 164	145	
15½-20	5.5	153, 81, 177, 107, 166	137	
20½-25	5.8	165, 124, 157, 104, 188	148	152.0 ± 4.2
25½-30	6.0	175, 133, 109, 148, 143	142	
30½-35	6.0	155, 105, 139, 159, 162	144	
35½-40	6.1	153, 116, 150, 149, 173	148	
40½-50	6.2	185, 133, 151, 164, 164	161	
50½-60	6.3	210, 137, 126, 123, 196	158	
1-1½ hr.	6.5	170, 138, 186, 177, 207	176	
1½-2	6.6	158, 152, 177	162	
2-3	6.5	158, 136	147	

varying from 3 to 5 ml./l. (45-75% saturation) during the 10 min. that the activity is most apparent. Thus the failure of the carbon monoxide animals to increase their oxygen uptake with their increase in activity indicates that within this range of

oxygen pressures the haemoglobin is normally functional, enabling untreated larvae to pick up the extra oxygen demanded by increased activity; in the carbon monoxide larvae the energy for such activity must have been partly derived from anaerobic processes.

Ewer (1942) showed that in *Chironomus dorsalis* at 17° C. the haemoglobin of larvae only became functional at oxygen concentrations of 3 ml./l. (44% air saturation) or less; the above experiments indicate that the haemoglobin of my larvae was being used in oxygen transport at a slightly higher concentration. This difference is probably accounted for by the fact that with *C. plumosus* the increased oxygen

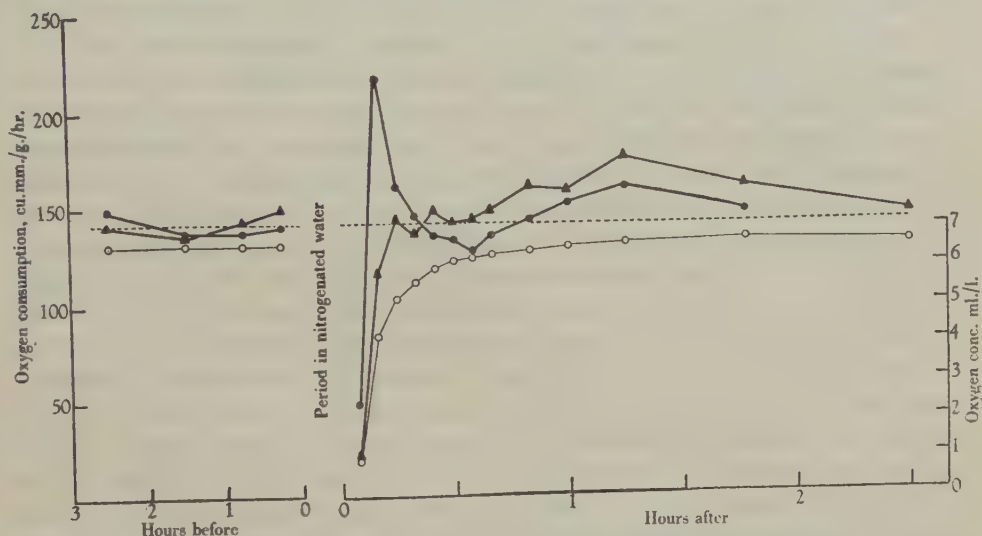


Fig. 4. Rates of oxygen consumption at 17° C. of *Chironomus plumosus* larvae before and after a 16 hr. period of anaerobiosis. September. ●, oxygen consumption of normal animals; ▲, oxygen consumption of carbon monoxide-treated animals; ○, oxygen concentration in respiratory vessel. The broken line represents the average metabolism of normal animals before nitrogen subjection. Data from Tables 3 and 4.

demands of the tissues during the period of great activity extend the range of external oxygen pressures at which the haemoglobin functions. The larger size of *C. plumosus* larvae may also intervene through their relatively smaller surface.

After the first 25 min. of recovery, in other words by the time that the larvae are once again in fully aerated water, the oxygen uptake of the carbon monoxide-treated larvae is equal to that of the untreated animals, and, as with them, is maintained for some time at a level slightly higher than the basal value. With the treated larvae this secondary increase was, in fact, greater than with the untreated animals and continued for a longer time, in some cases for 2 hr. after the beginning of recovery.

DISCUSSION

In all experiments described the larvae showed an increased metabolic rate during recovery from anaerobiosis. To what extent, however, this represents the repayment of an oxygen debt at 17° C. is uncertain, for the initial well-defined increase in

oxygen consumption is due to an increase in activity of the larvae, while statistical analysis of the subsequent more prolonged increase renders its reality doubtful. At 1° C., however, the rise in oxygen consumption lasting about 2 hr. is statistically significant and must be interpreted as the repayment of an oxygen debt. This may also be the case at 17° C. (without and with carbon monoxide), the statistical improbability being due to the small size of the rise.

It is possible from the experimental data to calculate the extent of such a debt in terms of cu.mm. oxygen consumed by recovering larvae over and above that which they would normally consume in the same time; thus in Figs. 2 and 3 this extra oxygen consumption is represented by the area between the curves of observed metabolic values and the lines drawn at the level of the basal metabolism of the larvae. The increased oxygen consumption of the winter and summer larvae at 17° C. amounts to 12.0 and 14.0 cu.mm. oxygen/g. respectively. Had these larvae not been subjected to anaerobic conditions the oxygen they would have consumed during the anaerobic period amounts to 2240 cu.mm. for the winter and 3080 cu.mm. for the summer larvae. The extra oxygen consumed during repayment of an oxygen debt is thus only 0.5% of that missed during the anaerobic period. Such a very small oxygen debt of *Chironomus* is in striking contrast to the debts accumulated by vertebrate tissues after anaerobic activity, in which the amount of increased oxygen consumption is proportional to the length of the anaerobic period.

The extent of repayment of oxygen debts among invertebrates, however, varies considerably. Well-defined debts have been recorded in *Periplaneta* and *Lumbricus* by Davis & Slater (1928), in *Planorbis* by Borden (1931), in grasshoppers by Bodine (1928) and in *Tenebrio*, *Cryptocercus* and *Zootermopsis* by Gilmour (1940a, b, 1941). There is evidence that in some of these animals a proportion of the organic acids is resynthesized to carbohydrate: in others a greater repayment of oxygen debt indicates their complete removal by oxidation. In yet other invertebrates oxygen debts are clearly not repaid in full; thus the ciliate *Tetrahymena geleii* only repays 25% (Thomas, 1942, cited in von Brand, 1945), and *Eustrongylides ignotus* 30% (von Brand, 1942) of the debts incurred. Both these animals are known to excrete organic acids and in this way lessen the need for repayment. Excretion of the waste products formed may also account for the small size and variability of the oxygen debts recorded in *Planaria* (Lund, 1921), *Nereis* (Hyman, 1932), *Urechis* (Hall, 1931) and *Tubifex* (Dausend, 1931; Harnisch, 1935, 1936). Finally, Harnisch (1942) could find no evidence of any repayment of oxygen debt in the larvae of *Chironomus bathophilus*.

Studies of the anaerobic glycogen metabolism of invertebrates which successfully withstand periods of anaerobiosis in nature have shown that various higher and lower fatty acids are formed by glycogen fermentation (for literature see von Brand, 1945). In intestinal parasites these fatty acids are either converted to non-toxic fat, and stored, or, more commonly, excreted by the animal. In their ability to excrete the products of anaerobic metabolism they are thus adapted to withstand prolonged periods of oxygen lack. That free-living animals which live in oxygen-poor surroundings should also be able to remove the products of carbohydrate fermentation

in this way is not surprising, their respiratory problems being of the same nature as those of internal parasites. In aquatic invertebrates the elimination of such waste products might be either by excretory organs, or in the case of small animals by diffusion across the surface of the body, provided that this is permeable to them. The repayment of oxygen debts by animals such as *Mya* and *Anodonta* (van Dam, 1938) is possibly made necessary by the shutting of the valves of the shell during anaerobiosis, which would limit the ready diffusion of waste products out of the body. The accumulation of toxic waste products within the body, and the need for oxygen to remove these in the repayment of an oxygen debt, is most apparent in animals least adapted to withstand oxygen lack, such as terrestrial invertebrates and the vertebrates.

It seems probable therefore that the absence of an oxygen debt at all proportional to the length of the anaerobic period in *Chironomus* larvae is due to the removal, either by diffusion or excretion, of the waste products as they are formed. With this in view the water in which a number of larvae had been kept anaerobically overnight was tested for lactic acid. This was found to be present. Although lactic acid is only one of several organic acids produced by the incomplete breakdown of glycogen, it at any rate was removed from the larvae as such and not retained in the body. The observed slight increase in oxygen consumption after anaerobiosis would thus account only for the transformation of those products which remained within the body owing to their indiffusability. In this case the greater repayment of debt by chilled animals could be accounted for by a greater accumulation of such products within the body due to a reduced rate of diffusion or excretion. The fatty acids formed during anaerobiosis may also to some extent be converted into fat within the body, as in certain endoparasites, and the need for repayment of oxygen debt further reduced in this way. Evidence supporting this is given by Harnisch (1939) who found that towards the end of a long anaerobic period (23–24 hr.) the production of fatty acids decreases and fat is formed in the bodies of the larvae.

It is interesting that Harnisch (1942) could find no evidence of repayment of oxygen debt after anaerobiosis in *Chironomus bathophilus*, a species closely related to *C. plumosus* and capable of living in oxygen-poor lakes. Harnisch interprets this absence of oxygen debt as indicating that the larvae respire anaerobically at all times, even when oxygen is present. It may, however, be that in this euroxybiotic species the mechanisms for the rapid elimination of metabolic waste products have been perfected.

Harnisch (1936) has recorded considerably greater repayment of debt in *Chironomus thummi* than I found in *C. plumosus*. The average metabolism of his normal animals lay between 260 and 278 cu.mm./g./hr. while that of animals after 15 hr. in nitrogen varied in different experiments from 368 to 444 cu.mm./g./hr. Thus there was a 41–59% increase in metabolic rate, persisting for two or more hours. Correlated with this increased metabolism he noted that the larvae showed regular undulatory movements which continued for many hours. Since larvae previously kept in oxygenated water did not show such activity he regarded the movements as indicating 'dass im Körper der Tiere Produkte anoxybiotischen Stoffwechsels

vorhanden sind und somit gesteigerter Sauerstoffverbrauch ("sekundäre Oxybiose") besteht'. An alternative interpretation, not suggested by Harnisch, is that the increased metabolism is a direct result of the increased activity rather than of an oxygen debt. The fact that he sometimes found larvae which had previously been kept in oxygenated water to be making vigorous respiratory movements, in which case the animals also showed a high oxygen consumption, supports this interpretation. Harnisch's increased oxygen uptake would then be parallel to that recorded during the short period of intense activity in my experiments. That his larvae remained active much longer than mine may be due to a specific difference. In view of his lack of appreciation of the activity factor, his results should be treated with caution.

Harnisch also conducted a series of experiments on carbon monoxide-treated larvae after oxygen lack. He found these larvae to have a lower oxygen consumption than untreated larvae at all oxygen pressures at and below air saturation of water, and from this he concluded that when their metabolism is raised in paying back an oxygen debt the haemoglobin is functional all the time. During preliminary carbon monoxide treatment, however, he notes a reduction in the activity of the animals, their typical undulatory movements being no longer apparent, which is perhaps not surprising since he used pure carbon monoxide! The differences which he subsequently records between treated and untreated animals may well be caused merely by this activity difference.

In my series of experiments with carbon monoxide-treated larvae the animals, without functional haemoglobin, were capable of maintaining a metabolic rate greater than normal in aerated water, so that the respiratory pigment is of no significance in paying back a small debt in aerated water. That my larvae with carboxyhaemoglobin should actually have a slightly higher oxygen consumption than normal larvae, appearing thus to pay back a greater debt, is curious; it can hardly be attributed to the fact that during their short activity period the movements were partly anaerobic, for any such additional debt incurred in only 10 min. must have been very small.

Harnisch's conclusion that after oxygen lack the larvae use their haemoglobin at all oxygen pressures is thus not confirmed by my experiments. The difference between our results may in part be due to the activity difference between the two species of larvae, the increased oxygen demand of the tissues of his active animals extending the range of function of the pigment so that it is used even at high oxygen pressures. During the short time that my larvae were most active the haemoglobin was functional, but this period coincided with a low oxygen pressure in the water, at which in any case the pigment would be used (Ewer, 1942). In both Harnisch's larvae and mine the possession of haemoglobin enabled the larvae to show an increased degree of activity aerobically.

The functional significance of the immediate violent activity of the larvae after anaerobiosis when water containing oxygen was introduced is only apparent when the animals are considered in their natural habitat. *Chironomus* larvae live in U-shaped tubes in mud and remain in their tubes during periods of oxygen lack.

Violent undulatory movements upon the reappearance of oxygen in the overlying water will have the effect of producing a current of water through the tube, thus both washing away any accumulated carbon dioxide or organic acids present in the tube and providing a continuous supply of water from which oxygen may be extracted. Since in nature the oxygen content of water previously depleted of oxygen is unlikely to rise as rapidly as in the experiments the maintenance of a ventilation current is important. Moreover, thanks to the haemoglobin, the undulatory activity necessary to set up such a current can be maintained aerobically in water containing little oxygen, enabling the animals to re-establish full aerobic metabolism as quickly as possible after a period of oxygen lack.

In this connexion I have now undertaken a study of the behaviour of larvae in their tubes in as natural conditions as possible. It may well be that their responses to oxygen lack in such circumstances are different from those under the very artificial experimental conditions described above.

SUMMARY

1. When *Chironomus plumosus* larvae receive aerated water after a period of anaerobiosis their oxygen consumption increases at once to a value well above the normal. This initial increase lasts for about 10 min. at 17° C., after which the oxygen consumption falls but continues to be slightly above normal for 1 hr.

2. The initial sudden considerable rise in oxygen consumption is due to increased activity of the larvae; it is absent in larvae made inactive by a low temperature of 1° C. Such larvae, however, maintain an oxygen consumption above the normal value for about 2 hr.

3. The increased oxygen consumption lasting for 1–2 hr. after anaerobiosis represents the repayment of an oxygen debt, but this debt is only 0.5% of the oxygen which would have been consumed had the anaerobic period been an aerobic one.

4. The small size of the oxygen debt suggests that most of the products of anaerobic metabolism are excreted.

5. The haemoglobin of the larvae does not function during the repayment in fully aerated water of the small oxygen debt. This is shown by experiments with and without carbon monoxide.

6. During the initial short considerable rise in oxygen consumption at 17° C. due to larval activity after anaerobiosis the haemoglobin is functional. This activity occurs while the water is not yet fully aerated, and so the haemoglobin enables the larvae to maintain their activity aerobically in water containing little dissolved oxygen.

7. In nature the undulatory activity of the larvae immediately after anaerobiosis must serve to ventilate the U-shaped tubes in which they live in the mud, and so to fill the tubes with aerated water.

I wish to thank Prof. H. Munro Fox, F.R.S., in whose laboratory this work was done, for advice and help.

REFERENCES

- BODINE, J. H. (1928). Insect metabolism. The anaerobic metabolism of an insect (Orthoptera). *Biol. Bull. Woods Hole*, **55**, 395.
- BORDEN, M. A. (1931). A study of the respiration and of the function of haemoglobin in *Planorbis corneus* and *Arenicola marina*. *J. Mar. Biol. Ass. U.K.* **17**, 709.
- BRAND, T. VON (1942). Physiological observations upon a larval *Eustrongylides*. *Biol. Bull. Woods Hole*, **82**, 1.
- BRAND, T. VON (1945). The anaerobic metabolism of invertebrates. *Biodynamica*, **5**, 165.
- DAM, L. VAN (1938). *On the Utilization of Oxygen and Regulation of Breathing in some Aquatic Animals*. Groningen.
- DAUSEND, K. (1931). Über die Atmung der Tubificiden. *Z. vergl. Physiol.* **14**, 557.
- DAVIS, J. G. & SLATER, W. K. (1928). The aerobic and anaerobic metabolism of the common cockroach (*Periplaneta orientalis*), III. *Biochem. J.* **22**, 331.
- EDWARDS, F. W. (1929). British non-biting midges (Diptera, Chironomidae). *Trans. Ent. Soc., Lond.*, **77**, 279.
- EWER, R. F. (1942). On the function of haemoglobin in *Chironomus*. *J. Exp. Biol.* **18**, 197.
- FOX, H. M. & WINGFIELD, C. A. (1938). A portable apparatus for the determination of oxygen dissolved in a small volume of water. *J. Exp. Biol.* **15**, 437.
- GILMOUR, D. (1940a). The anaerobic gaseous metabolism of the roach *Cryptocercus punctulatus* Scudder. *Biol. Bull. Woods Hole*, **79**, 297.
- GILMOUR, D. (1940b). The anaerobic gaseous metabolism of the termite *Zootermopsis nevadensis* Hagen. *J. Cell. Comp. Physiol.* **15**, 331.
- GILMOUR, D. (1941). Anaerobic metabolism in the larvae of *Tenebrio molitor* L.: gaseous metabolism and changes in glycogen, sugar, fat and lactic acid. *J. Cell. Comp. Physiol.* **18**, 93.
- HALL, V. E. (1931). The muscular activity and oxygen consumption of *Urechis caupo*. *Biol. Bull. Woods Hole*, **61**, 400.
- HARNISCH, O. (1935). Versuch einer Analyse der Sauerstoffverbrauchs von *Tubifex tubifex* Müll. *Z. vergl. Physiol.* **22**, 450.
- HARNISCH, O. (1936). Primäre und sekundäre Oxybiose der Larve von *Chironomus thummi*. *Z. vergl. Physiol.* **23**, 391.
- HARNISCH, O. (1939). Studien zum Anaeroben und Erholungstoffwechsel der Larve von *Chironomus thummi*. II. Säurebildung und 'Notoxybiose'. *Z. vergl. Physiol.* **27**, 275.
- HARNISCH, O. (1942). Zur Analyse der Oxybiose der Larve von *Chironomus bathophilus* K., einer *Chironomus*-Larve der Seentiefe. *Zool. Anz.* **139**, 1. (Seen in abstract only.)
- HYMAN, L. H. (1932). Relation of oxygen tension to oxygen consumption in *Nereis virens*. *J. Exp. Zool.* **61**, 209.
- KEILIN, D. (1929). Cytochrome and respiratory enzymes. *Proc. Roy. Soc. B*, **104**, 206.
- LUND, E. J. (1921). Oxygen concentration as a limiting factor in the respiratory metabolism of *Planaria agilis*. *Biol. Bull. Woods Hole*, **41**, 203.
- THOMAS, J. O. (1942). The anaerobic carbohydrate metabolism of *Tetrahymena geleii*. Dissertation, Stanford University.
- WARBURG, O. (1926). Über die Wirkung des Kohlenmonoxyds auf dem Stoffwechsel der Hefe. *Biochem. Z.* **177**, 471.
- WOLSKY, A. (1938). The effect of carbon monoxide on the oxygen consumption of *Drosophila melanogaster* pupae. *J. Exp. Biol.* **15**, 225.

THE FUNCTION OF HAEMOGLOBIN IN *TANYTARSUS* (CHIRONOMIDAE)

By BARBARA M. WALSHE

Zoology Department, Bedford College, University of London

(Received 13 May 1947)

(With One Text-figure)

Eighty years ago Lankester (1867) identified spectroscopically the red blood pigment of the larvae of *Chironomus plumosus* as haemoglobin. Subsequently (1873) he correlated its presence with the fact that they inhabit stagnant ponds and putrescent mud where the amount of accessible oxygen must often be small. Since then it has been generally assumed that their haemoglobin enables the larvae to live in surroundings deficient in oxygen, and experimental evidence for this has lately been supplied by Harnisch (1936) and by Ewer (1942). These workers compared the metabolism of normal larvae with that of larvae whose haemoglobin had been made functionless by conversion to carboxyhaemoglobin. They found that the pigment was only used as an oxygen carrier at low pressures of oxygen; it is used to the greatest extent in water which is 22 % saturated with air at 17° C. Ewer also made a series of measurements of the oxygen content of the pond from which her larvae were obtained and found oxygen concentrations corresponding to 32 % air saturation or less for periods of at least 16 consecutive hours. Thus it may be assumed that in nature the haemoglobin of the species with which Ewer worked is of functional value.

The larvae of several chironomid genera other than *Chironomus*, however, contain haemoglobin, and it has frequently been pointed out (Malloch, 1915; Harnisch, 1930) that the possession of haemoglobin by a larva is no proof that it can live in situations poor in oxygen. Thus, although *C. plumosus* is known to be very euroxybiotic the almost identical *C. bathophilus* is less so, while *Tanytarsus* species are markedly sensitive to low oxygen concentrations and only occur in well-aerated waters (Thienemann, 1923). The wealth of limnological data on the distribution of chironomids provides many other instances of stenoxymbiosis in larvae with haemoglobin. In such forms the haemoglobin can scarcely function in the same manner as it does in the *Chironomus* species studied by Harnisch and by Ewer, otherwise they would be capable of maintaining a normal aerobic metabolic rate at low oxygen concentrations. The functional significance of the haemoglobin of such oxygen-needy chironomids has never been satisfactorily studied. Harnisch (1930, 1933, 1937) made a comparative study of *Chironomus*, *Prodiamesa* and *Tanytarsus*, three chironomid genera with different capacities of withstanding low oxygen concentrations in nature, but he was mainly concerned with their respiration after periods of anaerobiosis and did not attempt to assess the mode of functioning of the haemoglobin in the stenoxymbiotic species.

I therefore chose *Tanytarsus* larvae for an experimental study of the function of the haemoglobin. These small larvae, which contain sufficient haemoglobin to make them red in colour, are very sensitive to oxygen lack and occur in nature in oligotrophic lakes, the oxygen content of which never falls below about 50% air saturation and in running water. The principle and techniques employed were those of Ewer (1942): the rates of oxygen uptake of normal larvae at different oxygen concentrations were compared with those of larvae whose haemoglobin had been converted into carboxyhaemoglobin and was thus incapable of transporting oxygen.

Final instar larvae (7–8 mm. in length) of *Tanytarsus brunnipes* (Zett.) were collected from a small stream in Cambridge. The species was determined from adults which emerged in the laboratory, the identification being checked by comparison with British Museum specimens. Larvae were collected a day or two before experiments and were kept in running aerated water in shallow dishes with a thin layer of mud.

The method used to determine the metabolic rate was that described by Ewer (1942), with minor modifications. Larvae were enclosed in 10 ml. glass syringes in distilled water buffered with sodium bicarbonate (normality 0.004). A small glass bead was put into each syringe to ensure adequate mixing of the water. Four syringes were used at a time, each containing between fifteen and twenty *Tanytarsus* larvae. They were clipped on to a large wheel rotating in a thermostatic water bath at 17° C. At intervals of approximately 1 hr. a sample of water was withdrawn from each syringe and its oxygen content determined by the syringe-pipette micro-Winkler method described by Fox & Wingfield (1938). Each experiment lasted about 3 hr.; during this time the pH of the water altered less than 0.2 unit, and the oxygen concentration fell from air saturation (6.75 ml./l.) to about 2 ml./l., or, in experiments at a lower range of oxygen contents, from 4 ml./l. to about 0.7 ml./l. At the end of the experiment the animals were removed from the syringes, dried on filter-paper and weighed. The oxygen consumption for each hour of the experiment was calculated in cubic millimetres of oxygen per gram wet weight per hour. The average of the oxygen contents of the water at the beginning and end of each hour's interval gave the oxygen content to which the oxygen consumption rate was referred.

The method of treatment with carbon monoxide was as follows. The animals were kept in a 170 ml. bottle in aerated water to which sufficient carbon monoxide-saturated water had been added to make the pressure of carbon monoxide one-sixth of that of the dissolved oxygen. They were then left in the dark until their haemoglobin was entirely converted into carboxyhaemoglobin. The conversion was tested spectroscopically and was found to require 1 hr. It was calculated that the removal of oxygen by the larvae during this time did not increase the relative pressure of carbon monoxide to more than one-fifth. Experiments using animals with carboxyhaemoglobin were made in water containing 0.2 ml./l. carbon monoxide to prevent dissociation of the carboxy-compound. At the end of these experiments the blood was tested spectroscopically to ascertain that the haemoglobin was still in the form of carboxyhaemoglobin; this was judged by the failure of the absorption bands to fade on the addition of sodium hydrosulphite. Experiments with carbon monoxide-

treated animals were alternated with those using normal animals. This ensured that larvae in the same physiological state were compared, since work on *Chironomus* (Walshe, 1947) had shown that seasonal differences in metabolic rate occur.

The utilization of haemoglobin at very low oxygen pressures was also tested in another way: the times of death of larvae with and without haemoglobin in water of low oxygen content were compared. Larvae were enclosed in 1 l. bottles containing water of known low oxygen content (ranging from 9 to 23 % air saturation) and kept in the dark at room temperature, the numbers of dead being noted at intervals. The experimental bottles were set up in pairs: one bottle with normal untreated larvae and the other with larvae previously treated with carbon monoxide, as already described. In all experiments except one (Exp. 3, Table 3) ten larvae were used in each bottle; in Exp. 3, twenty larvae were used. At the end of each experiment the oxygen content of the water was estimated.

The metabolic rate of *Tanytarsus* at 17° C. at various concentrations of dissolved oxygen is given in Table 1 and Fig. 1. The mean rate of oxygen consumption in fully

Table 1. *Oxygen consumption of Tanytarsus brunnipes larvae at 17° C. at various concentrations of dissolved oxygen*

Oxygen concentration (ml./l.)	Oxygen consumption (cu.mm./g. (wet weight)/hr.)	
	Separate values	Mean and s.e.
6.00-5.01	630, 507, 590, 494, 532, 528, 424, 522, 541, 544, 469, 552, 489, 553, 454	525 ± 13
5.00-4.01	343, 440, 442, 435, 117, 322, 305, 231, 299, 424, 358, 450, 415	352 ± 27
4.00-3.01	505, 435, 424, 421, 415, 376, 435, 73, 51, 101, 326, 288, 344, 318, 367, 352	327 ± 34
3.00-2.01	309, 362, 316, 248, 223, 357, 298, 240, 354	301 ± 18
2.00-1.61	229, 246, 319, 330, 334, 379, 316, 219, 295, 228, 183	279 ± 18
1.60-0.90	224, 194, 224, 262, 273, 357, 299, 271, 298	267 ± 16

aerated water is 525 ± 13 cu.mm./g. (wet weight)/hr., but falls considerably at lower oxygen values. It is seen from the curve that the metabolism is dependent on the oxygen pressure in the water at all pressures below that corresponding to air saturation. This is contrary to Harnisch's statement that the oxygen consumption of *Tanytarsus* is constant over a wide range of oxygen pressures and only begins to decline at the low pressure of 1-2 % of oxygen (equivalent to 7 % air saturation) (Harnisch, 1929). The data from which he draws this conclusion, however, are so badly presented in his paper that it is impossible to estimate their validity. Using a Warburg manometer he plots the decline in pressure in the apparatus caused by the oxygen consumption of the larvae in a series of graphs lacking both ordinates and abscissae, and he judges the effects on the larvae of various gas mixtures by changes in the rate of decrease of the gas pressure. He selects certain experiments as examples for discussion and gives no idea of the actual numbers of experiments made, or of the range of gas mixtures used. The temperature for one experiment was 'etwa 20° C.', that of the others is not given. In comparing the effect of low oxygen

pressures on *Eutanytarsus inermipes* (= *Tanytarsus brunnipes*) with *Chironomus* he states: 'Ich habe leider keinen Versuch, der die Stelle klar trifft, an der *Eutanytarsus* vor *Chironomus* knickt. Sie wird sich aber noch finden lassen, da die Abknickung der Atmungskurve von *Eutanytarsus* stets deutlich stärker ist als die von *Chironomus thummi*.' This conclusion he subsequently quotes (1930) as an established fact.

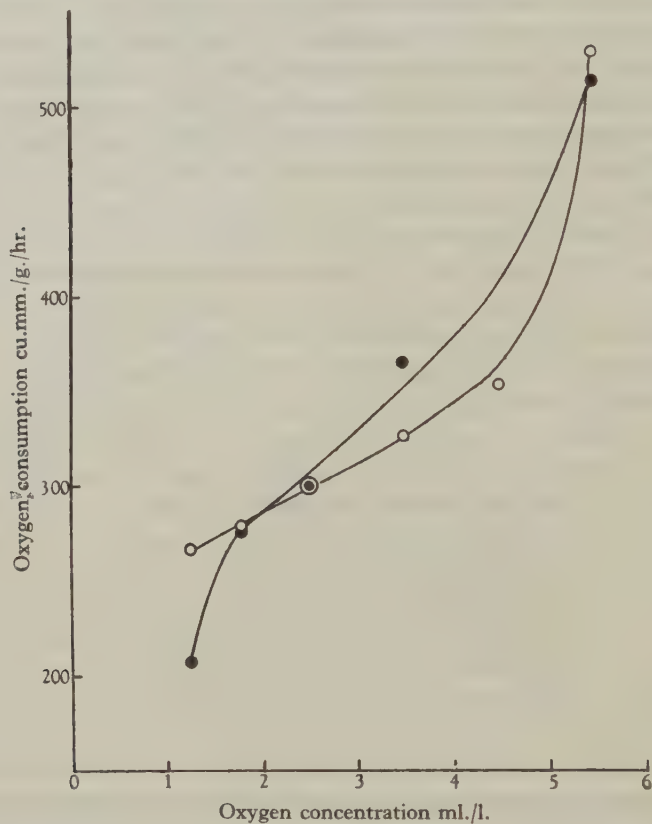


Fig. 1. Rates of oxygen consumption at 17°C. of *Tanytarsus* at various concentrations of dissolved oxygen. ○, normal animals; ●, animals with carboxyhaemoglobin. Data from Tables 1 and 2.

The general significance of dependence or independence of the oxygen consumption of animals on oxygen pressure has been very intensively discussed (Henze, 1910; Amberson, Mayerson & Scott, 1924; Rashevsky, 1933; Maloeuf, 1937*a, b*) and will not be elaborated here. Harnisch's theory (1937), however, that a dependent type of curve is an indication of a repayment of oxygen debt by the animals cannot be the explanation in the case of my larvae. In order to avoid the possibility of the accumulation of an oxygen debt before experiments, Harnisch kept his experimental animals in running water in wide glass tubes with bolting silk ends. I repeated this technique in a number of my experiments, but the subsequent

metabolism of larvae so treated was still just as dependent on the oxygen content of the water as without this treatment.

The oxygen consumption at various concentrations of dissolved oxygen of *Tanytarsus* larvae with carboxyhaemoglobin is given in Table 2 and Fig. 1. As in the case of the untreated animals, the oxygen consumption is high in fully aerated water and falls with declining oxygen pressures. A statistical comparison of the metabolic rates of treated and untreated larvae shows that between 6.0 and 1.6 ml./l. oxygen (90%–24% air saturation), the slight differences recorded are not significant. Below 1.6 ml./l. the oxygen consumption of carbon monoxide-treated larvae drops to 78% that of normal larvae: this decrease is statistically significant. It indicates that below this oxygen value the haemoglobin is functional in the normal animal in picking up oxygen.

Table 2. *Oxygen consumption of Tanytarsus brunnipes larvae with carboxyhaemoglobin at 17° C. at various concentrations of dissolved oxygen*

Oxygen concentration (ml./l.)	Oxygen consumption (cu.mm./g. (wet weight)/hr.)		
	Separate values	Mean and s.e.	% normal rate
6.00–5.01	513, 524, 424, 622, 394, 547, 547, 455, 689, 531, 471, 478, 630, 663, 412, 283, 639, 411, 369	505 ± 25	96
5.00–4.01	564, 509, 451, 504, 412, 356, 345, 254, 336, 442, 265, 370, 299, 419, 273	387 ± 25	109
4.00–3.01	322, 564, 370, 286, 498, 408, 421, 605, 477, 167, 360, 127, 169, 376, 448, 235	365 ± 25	112
3.00–2.01	427, 494, 246, 291, 313, 358, 277, 249, 307, 271, 212, 168	301 ± 27	100
2.00–1.61	287, 285, 222, 268, 271, 228, 370, 244, 368, 216	276 ± 17	99
1.60–0.90	178, 206, 238, 204, 273, 163, 241, 205, 191, 330, 163, 190, 204, 126	208 ± 14	78

The function of the haemoglobin at very low oxygen pressures was confirmed and extended by experiments of another type. The reason for the different technique was as follows. The experiments in syringes were unsuitable for detecting the slight differences in metabolic rate between untreated and treated larvae at very low oxygen pressures for the following reason: unless the differences were considerable they might easily be masked at oxygen concentrations of less than about 1 ml./l. by the random error of the Winkler method. This error, being largely determined by the titration end-point and by the introduction of oxygen in the reagents, is independent of oxygen concentration and is therefore relatively greater at low concentrations. For this reason another method was adopted and the times of death of larvae with and without carboxyhaemoglobin in waters of low oxygen contents were studied. A more rapid death of larvae with carboxyhaemoglobin was taken to mean that the haemoglobin of normal larvae functions at that oxygen pressure. The data from a number of such experiments are summarized in Table 3. Exps. 1 and 6 were made in winter at a lower temperature than the others and the absolute values for survival are thus

not comparable throughout the series, but since experiments with and without haemoglobin were always made simultaneously the assessment of the function of the haemoglobin remains valid. Larvae without functional haemoglobin kept in water with an initial oxygen content of less than 15% air saturation (approximately 1 ml./l.) died more rapidly than normal larvae (Exps. 1-3). It follows that at this low oxygen concentration the haemoglobin is of survival value to the larvae.

A more rapid rate of death of carbon monoxide-treated larvae also occurred when the initial oxygen content of the water was greater than 15% saturation (Exps. 4-6), but the increased rate of dying was not apparent in these larvae until many hours after the start of the experiment. Although the larvae were enclosed in a relatively

Table 3. *Rates of death of Tanytarsus brunnipes larvae at low oxygen concentrations*

Exp. no.	Temp. (° C.)	Larvae with haemoglobin				Larvae with carboxyhaemoglobin			
		Oxygen, % air saturation		Hr. after enclosure	% alive	Oxygen, % air saturation		Hr. after enclosure	% alive
		Initial	Final			Initial	Final		
1	13-14	9	7	44	90	9	8	44	80
				52	90			52	70
				68	80			68	40
				75	80			75	20
2	18-20	12	6	21	100	12	6	21	100
				28	100			28	70
				50	100			50	0
3	18-20	14	5	20	100	14	8	20	100
				45	95			45	0
4	18-20	15	11	20	100	15	12	20	100
				27	100			27	100
				43	90			43	90
				49	90			49	60
5	18-20	20	15	21	90	22	15	21	100
				28	90			28	100
				50	90			50	60
6	13-14	23	15	44	100	25	15	44	100
				52	100			52	100
				68	90			68	80
				75	90			75	70
				97	80			97	40

large volume of water their metabolism appreciably reduced its oxygen content during the course of the experiment (as is shown in Table 3) and it is therefore not possible from these experiments to determine precisely the highest oxygen concentration at which the haemoglobin is functional. Since, however, the bottles were opened and their oxygen contents determined as soon as an increased rate of dying was apparent, the critical oxygen value at which the haemoglobin becomes of significance for survival would seem to be about 15% air saturation (1.1 ml./l. at 14° C., 0.9 ml./l. at 19° C.). Above 15% saturation it is possible that the haemoglobin is also of value in oxygen transport and therefore in ultimately increasing the length of survival, but that the fatal effects of a slightly inadequate oxygen supply take some hours to become apparent.

The lower limit of the range of oxygen pressures at which the haemoglobin functions can more easily be determined. To do so larvae were enclosed in water of low oxygen content in small specimen tubes and the state of their haemoglobin was observed with a spectroscope. When the haemoglobin became fully deoxygenated the oxygen concentration of the water was determined. The average oxygen concentration at which this occurred was 5% air saturation. At and below this concentration, therefore, their haemoglobin can have no functional value.

The range of oxygen pressures over which the larvae of *Tanytarsus* use their haemoglobin at 17° C. is thus from 5 to less than 25% air saturation, while that of *Chironomus* is from 9 to 37% air saturation (Ewer, 1942). The haemoglobin therefore functions at lower oxygen pressures in *Tanytarsus* than in *Chironomus*. In normal *Chironomus* larvae the oxygen uptake at 17° C. is constant and independent of the oxygen concentration of the water down to about 15% air saturation. Above 37% air saturation of the water the animal's oxygen requirements are met by oxygen in physical solution in the blood, but between 37 and 15% air saturation of the water the animal's oxygen uptake owes its independence of external oxygen pressure to a functional haemoglobin. The haemoglobin is thus of functional value to the animal in making available enough oxygen for normal metabolism at oxygen pressures too low to allow of this in the absence of haemoglobin.

In *Tanytarsus*, however, it is meaningless to talk of the normal oxygen requirements of the larvae, since their oxygen uptake varies with the external oxygen pressure and one cannot therefore say what is normal. Even when the haemoglobin is functioning at low oxygen pressures it only raises the metabolic rate a little compared with larvae lacking haemoglobin. The haemoglobin still leaves the oxygen uptake considerably below that of animals in higher oxygen pressures. If a dependence of oxygen uptake on oxygen pressure indicates inadequacy of oxygen transport to the tissues (Henze, 1910), then at all oxygen concentrations below air saturation* *Tanytarsus* suffers partial oxygen lack and the haemoglobin does nothing to alleviate this condition except at very low oxygen pressures, and even then the extra oxygen picked up by the haemoglobin only slightly increases the metabolic rate. The tissues of *Tanytarsus*, in fact, have such a high oxygen demand that they suffer oxygen lack at oxygen pressures of the blood far higher than those which will cause dissociation of a haemoglobin with a high oxygen affinity, such as that of *Tanytarsus*; the oxygen linked with the haemoglobin cannot be of use to the oxygen-greedy tissues.

Is it, however, legitimate to assume that at all oxygen pressures at which the oxygen uptake is dependent the animal is necessarily suffering real oxygen shortage? It may be that animals with this type of metabolism have a surfeit of the intracellular oxidation-reduction systems, enabling them to show a high metabolic rate when plentifully supplied with oxygen, but not necessarily being adversely affected at lower oxygen pressures by an incapacity to maintain this rate. This hypothesis could only be tested by determining the lowest oxygen pressure at which such an animal could live indefinitely and normally. This, to my knowledge, has never been

* And possibly above it. My data, not extending above air saturation, do not settle this point.

done. It is known that *Tanytarsus* is very sensitive to poorly aerated water and Thienemann (1928) says that it is not found in nature in water below 50% air saturation. This is well above the upper limit at which the haemoglobin is used. In fact, the haemoglobin of *Tanytarsus* functions by transporting to the tissues an inadequate amount of oxygen, at an external oxygen pressure which in any case is probably ultimately lethal. The most it can do is to delay death a little. From this it is tempting to conclude that the haemoglobin plays no significant part as an oxygen carrier in the life of *Tanytarsus* in nature, in which case its presence may perhaps be due to some quality other than its oxygen transporting ability.

It should, however, be remembered that the animal lives in conditions very different from those presented to it in these experiments. *Tanytarsus* lives in mud tubes in well-aerated water, but nothing is known of the range of oxygen concentrations it actually encounters in these tubes, nor of its metabolic rate or response to declining oxygen pressures under these conditions.*

Evidence of this nature must come before an evaluation can be made of the normal significance of the haemoglobin of *Tanytarsus*.

SUMMARY

1. The metabolic rate of *Tanytarsus* larvae is higher in air-saturated water than at lower oxygen concentrations; the oxygen consumption is thus dependent on the external oxygen pressure.

2. The haemoglobin in the blood of the larvae does not function in oxygen transport when the larvae are in water which is between 25 and 100% saturated with air at 17° C. Below 25% air saturation of the water the metabolic rate of larvae without functional haemoglobin (i.e. treated with carbon monoxide) is lower than that of normal larvae. When kept in water below this oxygen concentration larvae with carboxyhaemoglobin also die quicker than normal larvae.

3. The external oxygen concentration at which the haemoglobin in the blood of the larvae becomes deoxygenated is 5% air saturation.

4. The range of oxygen concentrations over which the larvae of *Tanytarsus* use their haemoglobin at 17° C. is thus from 5 to 25% of air saturation.

5. The doubtful significance of the haemoglobin in the life of *Tanytarsus* in nature is discussed.

This investigation was made in the Department of Prof. H. Munro Fox.

* Hyman (1932) records that *Nereis* in tubes has a lower metabolism, more independent of declining oxygen concentrations, than free animals.

REFERENCES

- AMBERSON, W. R., MAYERSON, H. S. & SCOTT, W. J. (1924). The influence of oxygen tension upon metabolic rate in Invertebrates. *J. Gen. Physiol.* **7**, 171.
- EWER, R. F. (1942). On the function of haemoglobin in *Chironomus*. *J. Exp. Biol.* **18**, 197.
- FOX, H. M. & WINGFIELD, C. A. (1938). A portable apparatus for the determination of oxygen dissolved in a small volume of water. *J. Exp. Biol.* **15**, 437.
- HARNISCH, O. (1929). Verbreitung und ökologische Bedeutung des Hämoglobins bei den Chironomidenlarven. *Verh. internat. Zoologenkongr. X. Budapest*, p. 345.
- HARNISCH, O. (1930). Daten zur Respirationsphysiologie Hämoglobin-führender Chironomidenlarven. *Z. vergl. Physiol.* **11**, 285.
- HARNISCH, O. (1933). Respirationsphysiologische Grundlagen der Ökologie der Chironomidenlarven. *Verh. dtsh. zool. Ges.* **35**, 209.
- HARNISCH, O. (1936). Primäre und sekundäre Oxybiose der Larve von *Chironomus thummi*. *Z. vergl. Physiol.* **23**, 391.
- HARNISCH, O. (1937). *Chironomus* und *Tanytarsus*. *Biol. Zbl.* **57**, 628.
- HENZE, M. (1910). Über den Einfluss des Sauerstoffdruckes auf den Gaswechsel einiger Meerestiere. *Biochem. Z.* **26**, 255.
- HYMAN, L. H. (1932). Relation of oxygen tension to oxygen consumption in *Nereis virens*. *J. Exp. Zool.* **61**, 209.
- LANKESTER, E. R. (1867). Preliminary notice of some observations with the spectroscope on animal substances. *J. Anat., Lond.*, **2**, 114.
- LANKESTER, E. R. (1873). A contribution to the knowledge of haemoglobin. *Proc. Roy. Soc.* **140**, 70.
- MALLOCH, J. R. (1915). The Chironomidae, or midges, of Illinois, with particular reference to the species occurring in the Illinois River. *Bull. Ill. Lab. Nat. Hist.* **10**, 275.
- MALOEUF, N. S. R. (1937*a*). Studies on the respiration (and osmoregulation) of animals. I. Aquatic animals without an oxygen transporter in their internal medium. *Z. vergl. Physiol.* **25**, 1.
- MALOEUF, N. S. R. (1937*b*). Studies on the respiration of animals. II. Aquatic animals with an oxygen transporter in their internal medium. *Z. vergl. Physiol.* **25**, 29.
- RASHEVSKY, N. (1933). Note on the mathematical theory of oxygen consumption at low oxygen pressures. *Protoplasma*, **20**, 125.
- THIENEMANN, A. (1923). Die beiden Chironomusarten der Tiefenfauna der Norddeutschen Seen. *Ver. int. Ver. Limnol. Kiel. Stuttgart*, **1**, 108.
- THIENEMANN, A. (1928). *Die Binnengewässer*. Stuttgart.
- WALSHE, B. M. (1947). On the function of haemoglobin in *Chironomus* after oxygen lack. *J. Exp. Biol.* **24**, 329.

THE SUCCINOXIDASE SYSTEM IN OYSTER MUSCLE

BY GEORGE FREDERICK HUMPHREY

Department of Biochemistry, University of Sydney, Australia

(Received 19 March 1947)

(With One Text-figure)

The importance of the role which the C_4 -dicarboxylic acids play in tissue respiration was pointed out by Szent-Györgyi in 1937. It is now known that succinic dehydrogenase is one of the few dehydrogenases which react directly with the cytochromes, and the 'succinoxidase' system has been investigated in a large number of vertebrate tissues. The action of succinate on the oxygen uptake of a few invertebrate tissues has been studied, but no detailed consideration has been given to the possible occurrence of a succinoxidase system. This system becomes especially important in view of the varied response of such tissues to agents like cyanide and azide, which have a marked effect on cytochrome oxidase.

The occurrence of cytochrome in lower organisms has not been widely investigated, although it is known that cytochrome oxidase is of frequent occurrence. Also, in the snail, there occurs heliocorubin (Dhéré & Vegezzi, 1917), a pigment whose chemical properties resemble those of cytochrome (Keilin, 1933). Baldwin (1938) found that in this animal, cytochrome occurs only in the radula.

In the present paper, some properties of the succinoxidase in oyster muscle have been investigated, particularly with reference to the action of inhibitors. The effect of the same inhibitors on the oxygen uptake of homogenates has been determined.

MATERIALS

The oysters used were 2-3 years' old specimens of *Saxostrea commercialis*, from the C.S.I.R. Experimental Farm at George's River, Sydney, Australia.

Cytochrome C was prepared from pig heart by the method of Keilin & Hartree (1937) but was dialysed against distilled water, following the recommendation of Potter (1941). The concentration was determined by measuring the absorption at $550\text{ m}\mu$ after oxidation with ferricyanide and reduction with hydrosulphite. The results for both forms agreed within 5%.

Sodium succinate was used as a neutralized ($\text{pH}=7.1$) solution of succinic acid. The inhibitors were similarly neutralized sodium salts.

METHODS

Homogenates were prepared as previously described (Humphrey, 1946) but at room temperature. The same manometric technique was employed. When cyanide was used as an inhibitor the centre-well contained 0.2 ml. of KOH-KCN solution (Krebs, 1935).

Inhibitors were always placed in the main compartment when preparing the flasks and would therefore be in contact with the enzyme for about 20 min. before the initial reading. The succinate was always tipped in from a side-arm. When added, cytochrome was present in a final concentration of $10^{-5} M$.

Succinoxidase was prepared from homogenates (200 mg./ml. in water). The suspension was diluted with an equal volume of water, centrifuged, and the residue stirred with water equal in volume to the discarded supernatant. After centrifuging again the residue was diluted with Sørensen phosphate buffer ($pH=7.5$) to give the same volume and a final phosphate concentration of $0.05 M$. The pH of this suspension is 7.1.

RESULTS

Effect of cyanide, azide and methylene blue

It can be seen from Table 1 that the muscle is very sensitive to cyanide, and also that azide in higher concentration depresses the oxygen uptake. The addition of a small amount of methylene blue can increase the activity of the uninhibited homogenate and also restore the activity inhibited by cyanide.

Table 1. *Effects of cyanide, azide and methylene blue on the whole homogenate*

Figures are percentage of control with no methylene blue or inhibitor. 600 mg. of muscle and $0.02 M$ (final concentration) phosphate in each flask. $pH=7.3$. 0.1 mg. methylene blue in last three flasks.

Cyanide	$10^{-3} M$	10
	10^{-4}	10
	10^{-5}	35
Azide	$10^{-1} M$	40
	10^{-2}	65
	10^{-3}	75
Methylene blue	—	130
	$10^{-2} M$ cyanide	100
	10^{-3} cyanide	98

These inhibitions suggest the presence of cytochrome oxidase. The results in Table 2 show that this enzyme is present.

Table 2. *Presence of cytochrome oxidase*

2 ml. succinoxidase preparation per flask; additions to give final concentrations of $10^{-5} M$ cytochrome, $10^{-2} M$ ascorbic acid (tipped in from side-bulb), $0.04 M$ phosphate. Final $pH=7.1$.

	$\mu l. O_2/hr.$
Enzyme	0
+ cytochrome	0
+ ascorbic acid	5
+ cytochrome + ascorbic acid	48
+ cytochrome + ascorbic acid + $10^{-2} M AlCl_3$	24
+ cytochrome + ascorbic acid + $3 \times 10^{-3} M AlCl_3$	12

Following the report of Horecker, Stotz & Hogness (1939), regarding the stimulating effect of aluminium in small concentration, Schneider & Potter (1943) recommend the use of this in cytochrome oxidase assays. However, the values in Table 2 show an inhibitory effect and therefore it was never used.

Detection of cytochrome

An attempt was made to estimate the amount of cytochrome in the muscle by a micro-isolation procedure (Potter & Dubois, 1942). After dissolving the final precipitate from 10 g. of muscle in 0.3 ml. NaOH, no bands could be observed with a Zeiss ocular micro-spectroscope, even after addition of hydrosulphite. Ground muscle added to benzidine-peroxide-acetic acid mixture have faint blue specks on the surface of the particles, indicating the presence of an oxidase, peroxidase or haem compound. The benzidine test was repeated as follows. Muscle was ground with 5*N* acetic acid, and the suspension extracted with ether; the ethereal layer was evaporated down and any residue dissolved in a little water. This solution was boiled, and when added as a top layer to the benzidine mixture, gave a definite blue-green ring.

The positive benzidine reaction suggests the presence of some haem compound. Also, it seems that cytochrome, if present, is in very small amount.

Oxidation of succinate

Increased oxygen consumption is observed in the presence of succinate (Table 3). This indicates that the system is quickly saturated with succinate, especially in the absence of cytochrome.

Table 3. *Oxidation of succinate*

Each flask contained 0.02 *M* phosphate and 600 mg. of muscle. Final pH = 7.3. Values are $\mu\text{l. O}_2/\text{hr.}$

Concentration of succinate	Cytochrome	Without cytochrome
—	22	15
0.01 <i>M</i>	60	30
0.02	83	33
0.05	85	35

Little loss of succinic dehydrogenase occurs during the preparation of the succinoxidase (Table 4).

Table 4. *Distribution of succinic dehydrogenase*

Each flask contained the equivalent of 500 mg. of muscle. Also cytochrome, 0.03 *M* succinate, 0.02 *M* phosphate and 5×10^{-3} *M* CaCl_2 .

	$\mu\text{l. O}_2/\text{hr.}$
Whole homogenate	94
First supernatant	38
Second supernatant	53
Succinoxidase	98

Calcium is included because of the observations of Keilin & Hartree (1940), Schneider & Potter (1943), and Axelrod, Swingle & Elvehjem (1941), demonstrating the relationship between calcium, oxalacetic acid, and diphosphopyridine nucleotide. Since Ochoa & Ochoa (1937) have found the latter compound in the muscles of

Ostrea edulis, it is not surprising that calcium is needed (Table 5) to give maximum activity. Fig. 1 gives a pH curve for the system, and Table 5 shows the changes in activity due to altered substrate concentration.

Table 5. *Effect of Ca and succinate concentration*

2 ml. succinoxidase suspension in phosphate buffer in each flask. Also cytochrome.

Succinate	CaCl ₂	μl. O ₂ /hr.
0.03 M	—	9
0.03 M	$5 \times 10^{-3} M$	27
0.03 M	$10^{-2} M$	18
—	$5 \times 10^{-3} M$	0
0.01 M	$5 \times 10^{-3} M$	19
0.02 M	$5 \times 10^{-3} M$	25
0.03 M	$5 \times 10^{-3} M$	27
0.10 M	$5 \times 10^{-3} M$	30

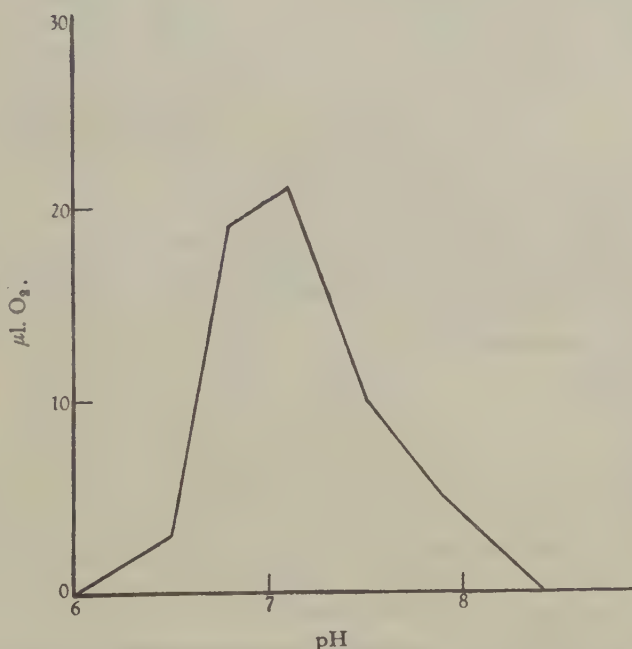


Fig. 1. The effect of pH on the oxygen uptake of the succinoxidase preparation. Each flask contained 2 ml. of succinoxidase; cytochrome, $5 \times 10^{-3} M$ CaCl₂ and 0.03 M succinate were also added. The figures represent μl. O₂/hr.

Effects with inhibitors

A number of inhibiting agents were tried on the succinoxidase suspension in the presence of 0.01 or 0.03 M succinate.

Apart from cyanide, selenite was the most powerful inhibitor of succinoxidase (Table 6), but arsenite was comparatively weak. On the whole homogenate (Table 7), arsenite had a much greater effect, and 0.01 M fluoride brought about a stimulation.

This is perhaps due to combination with inhibiting metals, or a shunting of the metabolism into another pathway (Borei, 1945). As in Table 1, azide is less effective than cyanide.

A comparison of the results in Tables 6 and 7 seems to indicate that the succinoxidase system is not the chief one responsible for the respiration of the muscle. The conditions with regard to substrate concentration and presence of co-factors are so different, however, that this suggestion must be interpreted with caution.

Table 6. *Effect of inhibitors on succinoxidase*

2 ml. succinoxidase per flask. Cytochrome, CaCl_2 ($5 \times 10^{-3} M$) also present. Figures are percentage inhibition observed over 2 hr.

Inhibitor	Succinate concentration					
	0.03 M			0.01 M		
Concentration (M)	10^{-1}	10^{-2}	10^{-3}	10^{-1}	10^{-2}	10^{-3}
Malonate	60	43	17	84	68	15
Iodoacetate	92	55	34	76	48	44
Pyrophosphate	87	53	15	78	63	23
Fluoride	57	8	0	75	30	0
Arsenate	55	6	10	18	0	0
Arsenite	29	29	29	51	35	21
Selenite	90	94	80	94	70	64
Azide	90	80	40	90	95	45
Cyanide	—	90	85	—	90	100
			80 ($10^{-4} M$)			82 ($10^{-4} M$)

Table 7. *Effect of inhibitors on the whole homogenate*

Each flask contained 600 mg. muscle in 0.02 M phosphate (pH=7.3). Figures are percentage inhibition observed over 2 hr.

Inhibitor concentration (M)	10^{-1}	10^{-2}	10^{-3}		
Malonate	40	24	22		
Iodoacetate	69	65	70		
Pyrophosphate	69	50	47		
Fluoride	45	—12	19		
Arsenate	85	60	48		
Arsenite	70	68	40		
Selenite	89	82	70		
Azide	60	35	25		
Cyanide	85	88	90	90 ($10^{-4} M$)	65 ($10^{-5} M$)

DISCUSSION

Only a few of the invertebrates have been examined for their ability to metabolize added succinate. Baldwin (1938) demonstrated that slices of hepato-pancreas from *Helix pomatia* showed increased respiration after the addition of succinate. Using the Thunberg technique with washed tissue suspensions, Ball & Meyerhof (1940) found succinic dehydrogenase in *Limulus polyphemus*, *Busycon canaliculatum*, *Homarus americanus*, *Loligo pealii* and *Venus mercenaria*. The activity was most pronounced in the heart and faint or doubtful in white and adductor muscles. Navez, Crawford, Benedict & Du Bois (1941), using the chopped heart of *V. mercenaria*, found that added succinate raised the oxygen utilization by 10%. Methylene blue gave 150%, a value which was not further increased by the addition of succinate. *Saxostrea* seems

to be much richer in succinic dehydrogenase and/or those systems which can metabolize succinate than the animals used by either Ball & Meyerhof or Navez.

Loew (1891) found that azide was toxic to various worms, crustaceans and molluscs. Since Keilin (1936), and later, Keilin & Hartree (1940) showed that azide was as effective as cyanide in preventing the oxidation of cytochrome by cytochrome oxidase, workers have tended to assume that these two inhibitors have more or less the same effect on tissues. However, a comparison of their effects shows differences which are not easily explained. It seems that among the lower animals, there occur all possible combinations of cyanide sensitive and insensitive, and azide sensitive and insensitive respiratory systems (Stannard, 1939; Spiegelman & Moog, 1945). Working with *Ostrea edulis*, Chapheau (1932) investigated the effect of KCN on the oxygen consumption of chopped muscle. The Q_{O_2} values he obtained, in the presence of 10^{-5} , 10^{-4} , and $10^{-3} M$ KCN, respectively, were: 0.20, 0.12 and 0.10. No control figure is given, and no KCN was added to the centre-well of the Warburg flasks which he used. From other values given in his paper, the control value can be estimated at 0.25; i.e. at a concentration of $10^{-3} M$ KCN, 40% of the respiration is cyanide stable. Navez *et al.* (1941), using 'high concentrations of KCN' on *Venus mercenaria* heart, obtained no more than 40% inhibition of oxygen uptake, even in the presence of *p*-phenylene diamine or succinate.

Table 7 shows that cyanide produces very marked inhibition with *Saxostrea* muscle; at $10^{-5} M$ KCN, only a third of the normal oxygen consumption is obtained. To cause a similar effect, $10^{-1} M$ azide must be used, and its efficacy drops sharply at lower concentrations; the difference in inhibition given by cyanide from 10^{-1} to $10^{-4} M$ cannot be regarded as significant. Unfortunately, azide could not be tried at a pH where it might be more effective (about pH 6.5) since the normal respiration is depressed at this pH (Humphrey, 1946). From Table 6, it can be seen that cyanide is a very powerful inhibitor of the succinoxidase system, and again, that azide gives similar effects at a higher concentration.

Fluoride appreciably inhibits the succinoxidase in high concentration, but the effect decreases rapidly with dilution (Table 6). These results contrast with those of Navez *et al.* (1941), who found that fluoride had little or no effect on succinate oxidation by *Venus*. The action on the homogenate is not so marked, and the accelerating effect cannot be satisfactorily explained. Stotz & Hastings (1937) found that 0.06 *M* fluoride inhibited the succinic dehydrogenase from beef-heart about 45% and the oxidase, only 16%. Borei (1945) states that the action of fluoride is a competition with cytochrome oxidase for the cytochrome, and that fluoride may form a fluorophosphoprotein complex with cytochrome.

Iodoacetate has a marked inhibitory effect on succinoxidase and also on the homogenate even at $10^{-3} M$. Again this contrasts with the work of Navez *et al.* (1941). Inhibition may be explained as due to reaction with —SH groups (Hopkins, Morgan & Lutwak-Mann, 1938).

Dixon & Elliott (1929) found that pyrophosphate inhibited liver respiration by 30–70%. Leloir & Dixon (1937) showed that of a number of dehydrogenases tested, succinic dehydrogenase was the only one affected. Stotz & Hastings (1937) could

find no effect on the dehydrogenase with $0.06M$ pyrophosphate, but obtained 38% inhibition of the succinoxidase. The action on oyster muscle is very strong and the succinoxidase activity is significantly decreased even at $0.01M$.

Szent-Györgyi (1930) showed that $0.002M$ arsenite inhibited the oxygen consumption of liver by 67%; there was no inhibition of succinate oxidation. Potter & Elvehjem (1937), working with chicken kidney, found 87% inhibition in the presence of $0.01M$ arsenite, while, in the case where succinate was added, arsenite caused 70% inhibition; using $0.01M$ selenite, they obtained 95% inhibition of both succinate oxidation and endogenous respiration. With a beef-heart preparation, Stotz & Hastings (1937) showed that while $0.02M$ selenite did not affect the oxidase, succinic dehydrogenase was completely inhibited. Navez *et al.* (1941) found no inhibition of *Venus* heart by selenite. Both arsenite and selenite strongly inhibit the respiration of oyster homogenates; with the succinoxidase preparation selenite is again very powerful, but arsenite has only a moderate effect.

Arsenate, according to Potter & Elvehjem (1937), inhibits respiration and succinate oxidation only 20%. The results in Table 6 do not conflict sharply with this, but those in Table 7 give a much higher inhibition, only 15% of the normal oxygen uptake remaining in the presence of $0.1M$ arsenate.

Quastel & Woolridge (1928) showed that $0.01M$ malonate completely inhibited succinic dehydrogenase in bacteria. With pigeon-breast muscle, Stare & Baumann (1936) obtained 25% inhibition of respiration in the presence of $0.007M$ malonate. With the same concentration of malonate, Potter (1940) found complete inhibition of succinoxidase from pig-heart. Thus, malonate has come to be regarded as a very efficient inhibitor of succinic dehydrogenase. Its effect on the whole homogenate (Table 7) is not very marked, whereas, with low succinate concentration, it exerts a marked inhibition on succinoxidase. This could indicate that only a small part of the normal respiration goes through succinic dehydrogenase.

Recently, Ball & Meyerhof (1940) have called attention to the presence of haem compounds in animals possessing haemocyanin as a respiratory pigment. They say: 'Why an animal should employ haemocyanin for a blood pigment and yet possess muscles rich in the pigment myoglobin is indeed puzzling.' No satisfactory investigation of the presence of haemocyanin in oysters seems to have been made. Boyce & Herdman (1897) found only two instances where the plasma acquired a faint blue tinge on exposure to oxygen. In 1899, Herdman & Boyce recorded similar observations and pointed out that this occurred only in specimens showing a green leucocytosis. Maloeuf (1937) cites *Ostrea* as an animal with no type of 'oxygen transporter in internal medium and isolated organs'. Earlier, MacMunn (1886) had observed a faint but definite histohaematin spectrum before and after treating various tissues of *O. edulis* with ammonium sulphide; this indicates the presence of a haem compound and is supported by the positive benzidine test given by *Saxostrea* muscle. The low intensity of these last two reactions shows that the concentration of cytochrome must be very small; and this is interesting when compared with the relatively strong cytochrome oxidase and succinoxidase activities. It may be that this aspect of the metabolism of *Saxostrea* muscle is similar to that

occurring in *Arbacia* eggs; here, Krahll, Keltch, Neubeck & Clowes (1941) said that 'it seems safe to conclude that cytochrome C cannot carry a significant fraction of the oxygen consumption'. The positive benzidine reaction observed in *Saxostrea* might indicate the haemochromogen precursor mentioned by Keilin (1929). It is interesting to note that in insects, brown muscles give a strong benzidine reaction, whereas white muscles give scarcely any (Keilin, 1925). It seems, then, that *Saxostrea* can be classified as an animal with no respiratory pigment and with a very small concentration of cytochrome or cytochrome precursor.

CONCLUSIONS

The large increase in respiration (about 300%) observed in the presence of 0.01 *M* succinate indicates that the muscle possesses very active systems for the metabolism of succinate. It is not possible, as yet, to say whether the increase is direct succinate oxidation or is brought about by the catalytic effect of succinate on metabolism as a whole. The small malonate inhibition may be regarded as an indication that the endogenous respiration does not proceed very much through the succinate system.

The inhibition brought about by cyanide is such as to indicate very little cyanide-stable respiration. There is little evidence to support the suggestion of Navez *et al.* (1941) that the lamellibranchs use a flavoprotein or glutathione metabolism in contrast to the cytochrome-cytochrome oxidase systems. The low concentration of cytochrome found is to be expected in a white muscle; this may be correlated with the lack of oxygen-transporting pigment in the blood, i.e. there is a low metabolism of the organism as a whole. The increased oxygen uptake observed in the presence of methylene blue also indicates a lack of carriers in the experimental system, and the small rate of respiration can be explained by the sessile nature of the animal.

SUMMARY

Succinoxidase was prepared from the adductor muscles of *Saxostrea commercialis* by homogenizing in water and suspending the washed pulp in phosphate buffer. The effect of various inhibitors on this succinoxidase and on the oxygen consumption of homogenates was determined.

The respiration of the whole homogenate is almost completely inhibited by cyanide, selenite, and arsenate. Azide, arsenite, pyrophosphate and iodoacetate also inhibit strongly. Malonate and fluoride inhibit only about 40%. Methylene blue and cytochrome increase the oxygen uptake, the former being able to offset the inhibition caused by cyanide. Succinate brings about a 300% increase in respiration.

Calcium but not aluminium is needed for the action of succinoxidase, which is almost wholly inhibited by cyanide, azide, iodoacetate, pyrophosphate and selenite. Malonate and fluoride cause marked inhibition, whereas arsenate and arsenite are relatively inactive.

Cytochrome oxidase was detected in the succinoxidase preparation by using ascorbic acid as reductant.

Although cytochrome could not be isolated, the presence of a haem compound was shown by a positive benzidine reaction in an ethereal extract of a suspension of the muscle in acetic acid.

REFERENCES

- AXELROD, A. E., SWINGLE, K. F. & ELVEHJEM, C. A. (1941). *J. Biol. Chem.* **140**, 931.
 BALDWIN, E. (1938). *Biochem. J.* **32**, 1225.
 BALL, E. G. & MEYERHOF, B. (1940). *J. Biol. Chem.* **134**, 483.
 BOREI, H. (1945). *Ark. Kemi Min. Geol.* 20 A, no. 8.
 BOYCE, R. & HERDMAN, W. A. (1897). *Proc. Roy. Soc.* **62**, 30.
 CHAPHEAU, M. (1932). *Bull. Soc. sci. Arcachon*, **29**, 85.
 DHÉRÉ, C. & VEGEZZI, G. (1917). *J. Physiol. Path. gén.* **17**, 44.
 DIXON, M. & ELLIOTT, K. A. C. (1929). *Biochem. J.* **23**, 812.
 HERDMAN, W. A. & BOYCE, R. (1899). *Lancs. Sea-fish Mem.* no. 1.
 HOPKINS, F. G., MORGAN, E. J. & LUTWAK-MANN, C. (1938). *Biochem. J.* **32**, 1829.
 HORECKER, B. L., STOTZ, E. & HOGNESS, T. R. (1939). *J. Biol. Chem.* **128**, 251.
 HUMPHREY, G. F. (1946). *Aust. J. Exp. Biol. Med. Sci.* **24**, 261.
 KEILIN, D. (1925). *Proc. Roy. Soc. B*, **98**, 312.
 KEILIN, D. (1929). *Proc. Roy. Soc. B*, **104**, 206.
 KEILIN, D. (1933). *Ergebn. Enzymforsch.* **2**, 239.
 KEILIN, D. (1936). *Proc. Roy. Soc. B*, **121**, 165.
 KEILIN, D. & HARTREE, E. F. (1937). *Proc. Roy. Soc. B*, **122**, 298.
 KEILIN, D. & HARTREE, E. F. (1940). *Proc. Roy. Soc. B*, **129**, 277.
 KRAHL, M. E., KELTCH, A. K., NEUBECK, C. E. & CLOWES, C. H. A. (1941). *J. Gen. Physiol.* **24**, 597.
 KREBS, H. A. (1935). *Biochem. J.* **29**, 1620.
 LELOIR, L. F. & DIXON, M. (1937). *Enzymologia*, **2**, 81.
 LOEW, O. (1891). *Ber. deutsch. chem. Ges.* **24**, 2947.
 MACMUNN, C. A. (1886). *Philos. Trans.* **177**, 267.
 MALOEUF, N. S. R. (1937). *Z. vergl. Physiol.* **25**, 43.
 NAVEZ, A. E., CRAWFORD, J. D., BENEDICT, D. & DU BOIS, A. B. (1941). *Biol. Bull. Woods Hole*, **81**, 289.
 OCHOA, S. & OCHOA, G. (1937). *Nature, Lond.*, **140**, 1097.
 POTTER, V. R. (1940). *J. Biol. Chem.* **134**, 417.
 POTTER, V. R. (1941). *J. Biol. Chem.* **141**, 775.
 POTTER, V. R. & DUBOIS, K. P. (1942). *J. Biol. Chem.* **142**, 217.
 POTTER, V. R. & ELVEHJEM, C. A. (1937). *J. Biol. Chem.* **117**, 341.
 QUASTEL, J. H. & WOOLRIDGE, W. R. (1928). *Biochem. J.* **22**, 689.
 SCHNEIDER, W. C. & POTTER, V. R. (1943). *J. Biol. Chem.* **149**, 217.
 SPIEGELMAN, S. & MOOG, F. (1945). *Biol. Bull. Woods Hole*, **89**, 122.
 STANNARD, J. N. (1939). *Amer. J. Physiol.* **126**, 196.
 STARE, F. J. & BAUMANN, C. A. (1936). *Proc. Roy. Soc. B*, **121**, 338.
 STOTZ, E. & HASTINGS, A. B. (1937). *J. Biol. Chem.* **118**, 479.
 SZENT-GYÖRGYI, A. (1930). *Biochem. J.* **24**, 1723.
 SZENT-GYÖRGYI, A. (1937). *Acta med. Szeged.* **9**, 1.

OBSERVATIONS ON THE EFFECT OF MICROCLIMATE ON BITING BY *AÈDES AEGYPTI* (L.) (DIPT., CULICID.)

By W. H. R. LUMSDEN*

From the Department of Entomology, London School of Hygiene and Tropical Medicine

(Received 1 January 1947)

(With Two Text-figures)

INTRODUCTION

A summary of the results of earlier workers has already been given by Seaton & Lumsden (1941), of whose work the present is a further extension. In that paper were reported investigations to determine the effects of age, fertilization and light on the avidity for a blood meal of female *Aedes aegypti* (L.). There remained to be worked out the effects of variations in temperature and humidity of the microclimate at the time of biting, with which the present work deals.

MATERIALS AND METHODS

The *A. aegypti* used were originally derived from West Africa in 1926 and have been maintained since then in the laboratory, the adult females being fed on rabbits. Stock eggs were allowed to develop at weekly intervals. The larvae were fed on a mixture of powdered dog biscuit and 'Bemax', air being bubbled continuously through the cultures to prevent scum formation. As pupae appeared they were transferred to bowls of clean water and hatching was allowed to take place in cages in an insectary at about 28° C. and 65% relative humidity (means of seven observations 27.7° C. and 66% relative humidity). No attempt was made to segregate female pupae, as it had been found previously by Seaton & Lumsden (1941) that fertilization did not affect subsequent avidity for a blood meal. The bowls were transferred daily to clean cages, so that all the adults in a given cage were known to have eclosed in the previous 24 hr. Material for experimentation was usually derived from three of these successive daily cages or *lots* each week. Females were removed in *batches* of 12-15 to net-ended glass cylinders 7 cm. long and 3.5 cm. in internal diameter. Usually six of these *batches* made up from each *lot*. The cylinders were placed end to end in glass tubes 47 cm. long and 4.5 cm. in calibre, whose ends were closed by rubber bungs carrying short pieces of glass tubing serving as inlets and outlets for a stream of air. The tubes were connected in series; an air current, humidified by bubbling through a capillary immersed in glycerol-water solution of specific gravity 1.106, was passed through the whole series at a rate of about 8 l./hr. The glycerol-water solution and the tubes were maintained in

* Medical Research Council Senior Fellow in Tropical Medicine.

a darkened incubator. The tubes were covered with opaque black paper so that the mosquitoes were in constant darkness, even when the incubator was opened. Records of temperature were kept by means of a thermograph, checked daily against a chemical thermometer, and humidity determinations of the outflowing air were made from time to time by means of a dewpoint hygrometer. The extreme temperatures recorded were 23.4 and 26.3° C. The aggregate degree-hours for each lot was recorded, covering an average period of 12 hr. in the insectary, for which the mean temperature was taken as 28° C., and 4 days, reckoned from noon to noon, under the accurately controlled and recorded conditions in the incubator. Temperatures were reckoned from 8° C., as this appears from the work of Lewis (1933) on longevity to be a fairly accurate estimate of the temperature threshold of *A. aegypti* adults. Humidity records varied between 75 and 87% relative humidity, the mean of ten random observations being 81.6%. Batches of mosquitoes were starved under these conditions in the incubator for 4 days; they were used for experiments when 96–120 hr. old, i.e. in the age group 5 of Seaton & Lumsden (1941). Seaton & Lumsden estimated the percentage mortality in that age group at about 12. The mortality in the present work was recorded and was generally slightly less, probably owing to better humidity control. Lots in which the mortality exceeded 10% were excluded, as it had been found previously that higher mortalities were associated with heterogeneous behaviour of batches at the time of feeding. Such higher mortality rates were met with on a few occasions, as, for example, when defects in the incubator control allowed the temperature to rise for periods above the range mentioned above.

The experimental chamber was of the same basic type as that used in previous work but differed in the following details. It was of slightly different dimensions; length 18 cm., internal diameter 4.5 cm., and the net diaphragm 8.5 cm. from the lower end. The thermometer was 1.5 cm. from the skin surface. A disk of asbestos sheet 3 mm. thick, with a central hole through which the mosquitoes had access to the skin, was added to the lower end of the chamber to reduce heat interchange between the chamber interior and the applied arm. The size of the biting aperture was increased to 500 sq.mm. The hygrometer used was of the dewpoint type described by Buxton (1931) and was immersed in the water-bath, the air current passing through it immediately before entering the chamber.

As before, glycerol-water solutions in flasks immersed in the water-bath were used to obtain high humidities; for low humidity work the flasks were removed, and air which had been passed through two towers of silica-gel was admitted direct to the copper coil and filter in the water-bath. The rate of the air current was about 28 l./hr., corresponding to a renewal of the total volume of air in the experimental chamber 1.6 times/min. When much of the work had been completed, an imperfect joint was discovered, allowing some leakage of air from the upper end of the experimental chamber and therefore upsetting the precise determination of the atmospheric humidity therein. Further series of experiments were run at both high and low humidities, and where the earlier results are quoted, a note is added to make this clear.

The experimental procedure was similar to that already described, except that in the case of low humidities it was impossible to determine the humidity of the air stream using ether evaporation as the sole method of cooling. The thimble of the dewpoint apparatus was therefore cooled by means of a freezing mixture. This method was cumbersome, and it was considered sufficient to carry it out twice, before and after the experimental series run at low humidities, the conditions of the drying towers remaining unaltered in the meantime. Maintenance of the dewpoint hygrometer at -7.25°C . for several minutes resulted in no visible deposition of dew. This indicated a maximum possible vapour pressure of 2.7 mm. Hg (Landolt-Börnstein, 1923). The values plotted on the graph (Fig. 1) for the low-humidity series, are therefore maximum values only. Temperature and humidities recorded for experiments all refer to the conditions at the level of the chamber thermometer, 1.5 cm. from the skin surface. They are closely representative also of the conditions in the part of the chamber above this level, but below it the skin surface inevitably causes modifications dependent upon the temperature difference between it and the chamber air.

The temperature gradients close to the skin surface in the apparatus under normal working conditions are shown in Fig. 2. They were worked out by means of the resistance-type skin thermometer described by Bourne (1946). This instrument operates on resistance changes, due to temperature, in a minute bead enclosed at the tip of a glass probe. The instrument is designed primarily for the estimation of surface temperatures, i.e. with the bead in contact with a medium of fairly high conductivity. Calibration was carried out against a standard chemical thermometer in a mercury-bath enclosed in a water-filled thermos flask. If the instrument is used, as in the present work, for the estimation of air temperature, possible errors due to slowness of heat interchange between the bead and the surrounding air must be considered. The period required for stabilization in air was found to be less than 3 min., and as readings were taken at 3-4 min. intervals and only when the apparatus gave a constant reading, errors from this source were eliminated. The current necessarily passed through the bead to determine its resistance might be expected to have some heating effect resulting in too high values in media of low conductivity. The effect was, however, found to be negligible; two pairs of observations, in mercury and air, at 17 and 37°C . respectively, differed by under 0.15°C ., and in both cases the value for air was actually below that for mercury. Eight temperature gradients near the skin were worked out, two at each combination of high and low temperature and high and low humidity. There was no consistent difference due to humidity, and the curves given in Fig. 2 are representative.

Mosquitoes were allowed to remain in the chamber usually for 3.0-5.5 min. before the arm was applied, thus allowing for replacement of the air in the chamber 5-8 times. Only on four occasions was the preliminary exposure longer, 7-11 min. Three of these were in the lowest temperature group of the high-humidity series when the small amount of dew deposited made reading of the hygrometer difficult. The other was in the 35°C . group of the same series. The mosquitoes were allowed a period of 5 min. access to the pronator aspect of the forearm, always of the same

individual, in each experiment. In all experiments the chamber interior was in darkness.

Statistical methods used follow those given by Simpson & Roe (1939). Standard errors are used throughout.

THE EFFECT OF PRELIMINARY EXPOSURE TO LIGHT

Seaton & Lumsden (1941) have shown that direct illumination at 0.5 m.c. of the skin area available to mosquitoes reduces almost by half the numbers biting. Tate & Vincent (1935) found that up to about 20° C. prolonged illumination induced about 70% of females of the English anautogenous race of *Culex pipiens* L. to gorge, whereas only 6% or less of those kept in the dark did so. This effect occurred also at 25° C. but was not so well marked. Light did not significantly influence three autogenous races in this respect. In view of these findings it was decided first to see whether, after 4 days in the dark at about 25° C., variations in light intensity during the period of transference from the dark of the tubes in the incubator to the dark of the experimental chamber exerted any influence on the numbers of females biting. For this purpose two parallel series of experiments were run; in one series transference took place in the ordinary room conditions of diffuse daylight, and in the other the mosquitoes were manipulated in the lowest light intensity at which the operation could be satisfactorily carried out. This was in fact very low; it was found that with partially dark-adapted eyes the mosquitoes could be quite easily dealt with when seen only as vague silhouettes against a pale background. Eight experiments were carried out after manipulation in ordinary diffuse daylight, six with mosquitoes of lot 70 and two with mosquitoes of lot 71. Seven experiments were carried out after manipulation in dim light, four with mosquitoes of lot 70 and three with mosquitoes of lot 71. The aggregate degree-hours for these lots were both 1795, and their mortalities were respectively 7.3 and 9.8% during the 4 days in the incubator. The means and standard errors of the temperature and humidity records of the two series of experiments, based on both the initial and end readings, were:

Diffuse light series: $25.01 \pm 0.16^{\circ}$ C. and $77.2 \pm 0.92\%$ relative humidity.

Dim light series: $25.05 \pm 0.20^{\circ}$ C. and $78.75 \pm 1.1\%$ relative humidity.

The humidity records are, however, inaccurate owing to the chamber defect noted above, but this affected both series, so that as far as the effect of preliminary manipulation is concerned the series are considered comparable.

In the series manipulated in diffuse daylight five mosquitoes fed in one batch, six in one, seven in four and eight in two, a total of eight batches. In the corresponding series manipulated in dim light four mosquitoes fed in one batch, six in three and seven in three, a total of seven batches. The mean numbers feeding for the two series were:

Diffuse light series: 6.14 ± 0.37 .

Dim light series: 6.875 ± 0.33 .

These means are not significantly different, $t = 1.38$ and $P > 0.1$. It is therefore concluded that as such a wide variation in light intensity during the period of

transference to the apparatus does not significantly affect the results the much smaller normal variations can also be neglected.

THE EFFECT OF MICROCLIMATE AT THE TIME OF BITING

All experiments were carried out with the chamber interior in darkness after transference of the mosquitoes in diffuse daylight. Two successive series of experiments were run, one at high and one at low humidity. The distribution of lots to experimental batches is shown in Table 1. To counteract possible variations in the behaviour of lots, batches from a given lot were distributed as much as possible throughout the temperature values tested. It was not, however, practicable to distribute batches from one lot to both humidity series, as the time involved in

Table 1. *Distribution of lots of female Aedes aegypti to experimental batches and percentage mortality and degree-hour aggregates*

Lot no.	Total no. of mosquitoes put up	Mortality (%)	Degree-hour aggregate	Humidity series	No. of batches used in temperature groups (° C.)						Total no. of batches used
					10.8-16.3	19.8-20.9	25.0-25.7	29.5-30.8	33.5-35.3	39.1-40.0	
105	64	4.7	1860	Low	1	1	1	1	1	—	5
106	79	3.8	1864		1	1	1	1	1	1	6
107	78	5.1	1871		1	1	1	1	1	1	6
113	82	4.9	1889		1	1	1	1	1	1	6
114	78	1.3	1879		—	1	1	1	1	2	6
119	65	4.6	1875	High	—	—	1	1	2	—	4
120	77	2.6	1889		—	1	1	1	1	1	5
126	26	3.8	1876		2	—	—	—	—	—	2
127	79	0.0	1907		—	—	2	2	—	1	5
128	65	9.2	1904		1	2	1	—	1	—	5
129	75	2.7	1919		1	1	—	1	1	1	5
133	65	4.6	1974		1	1	—	—	—	2	4

changing the humidity controls frequently and waiting for the apparatus to stabilize would have made working impossibly slow. There was, however, no difference in the treatment of the mosquitoes used in these different series with the exception that owing to warm weather, which occasionally resulted in room temperatures slightly above the chosen incubator temperature, there was a slight increase in the aggregate degree-hours for some of the lots in the high-humidity series. The values for all the lots are shown in Table 1, which also shows the percentage mortalities.

Table 2 and Fig. 1 show the results of both series of experiments. The mean temperatures for each group and their standard errors are calculated from the initial and end temperatures of each experiment. The means of corresponding temperature groups in the two series are close together with the exception of the lowest pair. The temperature and humidity values plotted in Fig. 1 are the mean values for each experiment in the case of temperature, but, as noted above, the humidity values in the low-humidity series are mean maxima. Initial and end relative humidity estimations were almost always close together; the greatest divergence was 13% and most were less than 5% apart.

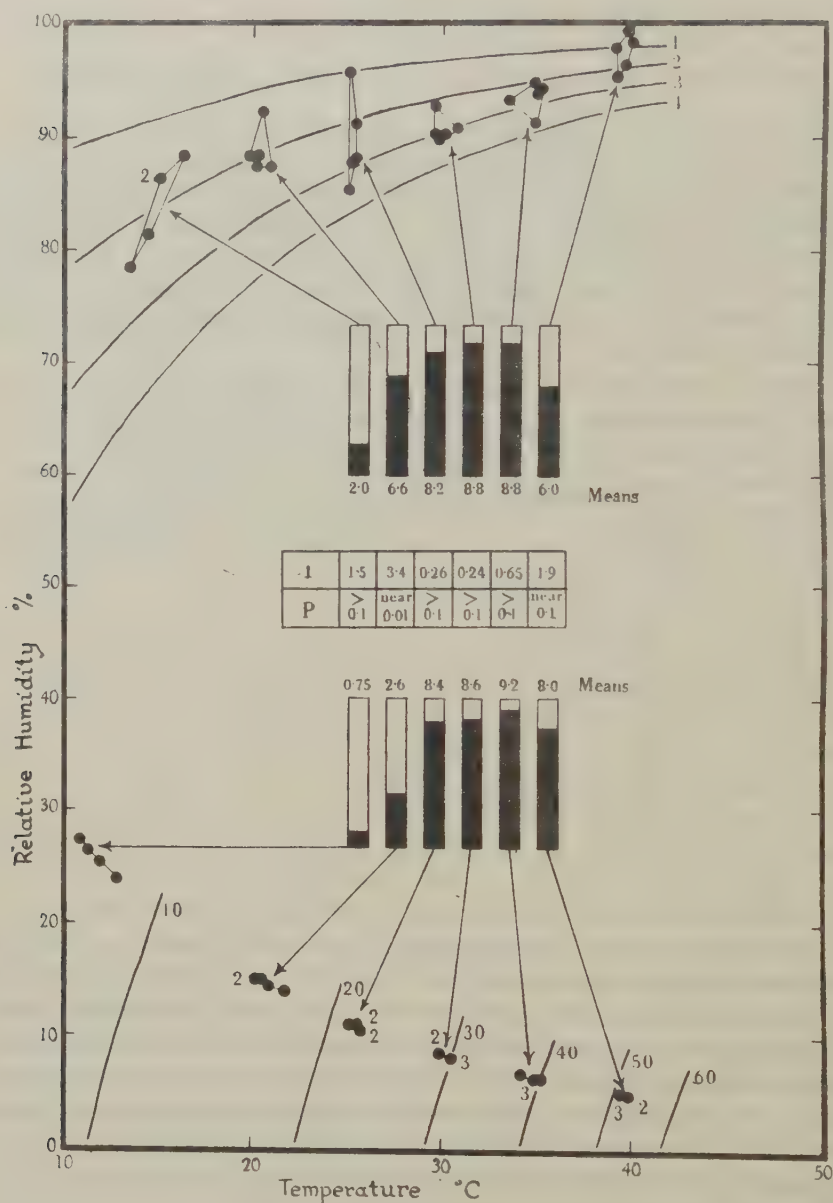


Fig. 1. Dots represent mean temperature and humidity conditions for individual experiments, each concerned with ten female *Aedes aegypti*. Arabic numerals adjoining dots indicate more than one experiment at identical mean conditions. Experiments treated together to give mean numbers feeding are joined into groups by lines. Histograms showing mean numbers feeding (Table 2) are connected by arrows to the groups of temperature-humidity values to which they refer. Curved lines are lines of equal saturation deficiency and are numbered with the value in mm. of Hg. Values of t and P are given in the central table for comparisons of the corresponding pairs of groups immediately above and below.

The main activity zone for feeding lies, in both series, from 25 to 35° C., with an optimum near 35° C. Humidity variations of wide amplitude appear to exert little influence, as only at 20° C. does the *t* test show any statistically significant difference between corresponding groups worked at high and low humidities. However, in view of the close correspondence of all the other groups, this difference may be regarded as fortuitous until it receives further confirmation. Lumsden & Bertram (1940) considered that the application of saliva to the skin of the fowls used for feeding *Aedes aegypti* females induced gorging, and at that time the apparent effect was thought probably to be due to a resultant rise in atmospheric humidity.

Table 2. *Numbers of Aedes aegypti females feeding in batches of ten, given 5 min. exposure to human arm, in darkness, at various temperatures and humidities*

Each + represents one batch, and the number of the column in which a given + falls indicates that that number of mosquitoes fed out of the ten comprising the batch.

Relative humidity series	Temp. mean and standard error (° C.)	Distribution of batches by no. of mosquitoes feeding											Mean no. feeding and standard error	Total no. of batches tested
		0	1	2	3	4	5	6	7	8	9	10		
High	14.8 ± 0.38	+	+	+	+	+	.	.	.	.	.	.	2.0 ± 0.63	5
	20.3 ± 0.30	.	.	.	.	.	++	+	.	+	.	.	6.6 ± 0.73	5
	25.2 ± 0.25	.	.	.	.	.	.	+	.	+	+++	.	8.2 ± 0.52	5
	30.0 ± 0.18	.	.	.	.	.	.	.	.	++	++	+	8.8 ± 0.33	5
	34.7 ± 0.23	.	.	.	.	.	.	.	.	+++	.	++	8.8 ± 0.44	5
	39.5 ± 0.14	.	.	.	.	.	+++	+	.	.	+	.	6.0 ± 0.70	5
Low	11.6 ± 0.41	+	+++	.	.	+	.	.	.	.	.	.	0.75 ± 0.22	4
	20.7 ± 0.35	+	.	++	.	+	.	.	+	++	+	+	2.6 ± 0.78	5
	25.4 ± 0.24	.	.	.	.	.	.	.	+	+	+	++	8.4 ± 0.46	5
	30.2 ± 0.26	.	.	.	.	.	.	+	.	+	+	++	8.6 ± 0.67	5
	34.8 ± 0.23	.	.	.	.	.	.	.	.	+	++	++	9.2 ± 0.33	5
	39.5 ± 0.14	.	.	.	.	.	.	+	+	+	+	+	8.0 ± 0.63	5

It was therefore unexpected to find in the present work that such wide variations in atmospheric humidity were without pronounced effect. Bishop & Gilchrist, similarly, in early work (1944) gained the impression that saliva increased the rate of gorging in mosquitoes offered a blood meal through animal membranes, but later (1946) proved that it had little effect. On the other hand, Lewis (1933) found a reduction in the proportion of *A. aegypti* feeding with lowered atmospheric humidity at both 25 and 30° C. His method was similar to that used in the present work, though there were several small differences in the treatment of the material. Lewis used females 6-7 days old kept in 'damp air' in the dark at 23° C.; his preliminary exposure was longer—10 min.; the area of skin per individual female was greater—about 240 sq.mm. instead of 50 sq.mm.; the chamber atmosphere was changed more slowly—only about 0.2 time/min.; sulphuric acid was used as the drying agent for low-humidity experiments. These differences are probably insignificant, but it is noteworthy also that his experiments were carried out in light, while in the present work the chamber interior was always in darkness. The

present work, and almost certainly that of Lewis also, is concerned with the first blood meal of *A. aegypti* females. It is generally agreed that in nature the first meal is taken in daylight though Marchoux, Salimbeni & Simond (1903) in South America consider that for later meals the mosquitoes tend rather to bite at night. Edwards (1941) quotes Flu in Surinam, and Cardamitis in Greece, as having come to the same conclusion. It is possible that the difference between the present results and those of Lewis may be due to different light conditions; perhaps low humidity as a factor inhibiting biting activity is only operative in the presence of light, since only during the day would the mosquito encounter humidities low enough seriously to endanger life. It is significant in this connexion that when Lumsden & Bertram (1940) obtained the impression that saliva encouraged gorging, light was not completely excluded. A second possible explanation is that in mosquitoes starved for long periods hunger may become a master factor causing activity in search of food despite unfavourable environmental conditions. Larsen (1943) observed several Noctuid moths on the wing by day following a period in which bad weather, particularly at night, had inhibited normal nocturnal activity.

The temperature gradients close to the skin in the present apparatus under normal working conditions are shown in Fig. 2. The lower curve, obtained with the apparatus at room temperature, shows that the temperature gradient is limited to the region within 10 mm. of the skin. The more gradual gradient of the upper curve must be attributed to slight cooling of the air during its passage down the chamber when working well above room temperature. The linear velocity of the air current in the chamber was about 0.5 cm./sec., which is well below the limit—30 cm./sec.—of the Beaufort Scale number 0 ('Calm') (*Meteorological Observer's Handbook*, 1926), so that a gradient closely similar to, or even steeper than, the lower curve must obtain under natural conditions. Both temperature and olfactory perception are generally accepted as being of importance in the location of the host by blood-sucking insects, and the available information has been summarized by Wigglesworth (1939). As far as mosquitoes are concerned there seems to be no information relating to the radius of action of the olfactory or other perception which must be responsible for the location of a host from a distance. It is fairly clear that perception of the host by temperature effects is limited to a few centimetres. One may cite in this connexion the work of Howlett (1910) with several mosquito species and Rivnay (1931) with *Cimex lectularius*. Wigglesworth & Gillett (1934), working with *Rhodnius prolixus*, considered that the effect was due to air temperature and not to radiant heat. The present work on the extent of the temperature gradient close to the skin corroborates the conclusion that temperature sense is a short-range one which must be responsible only for the final orientation of the insect and the eliciting of a probing response. Bishop & Gilchrist (1946), working with *Aedes aegypti* feeding through animal membranes, have adduced evidence that a temperature gradient steeply rising to the membrane was an important factor in attracting mosquitoes to feed. A higher rate of gorging was obtained when a gradient of from 24 or 28 to 42° C. was present than when both membrane and environment were at 37° C. Their work, as also the present work, was carried out with the mosquitoes

confined artificially close to the blood source where temperature would be expected to be the predominant factor. That *A. aegypti* females are able to locate their host at short range in the absence of a temperature gradient rising to the skin is, however,

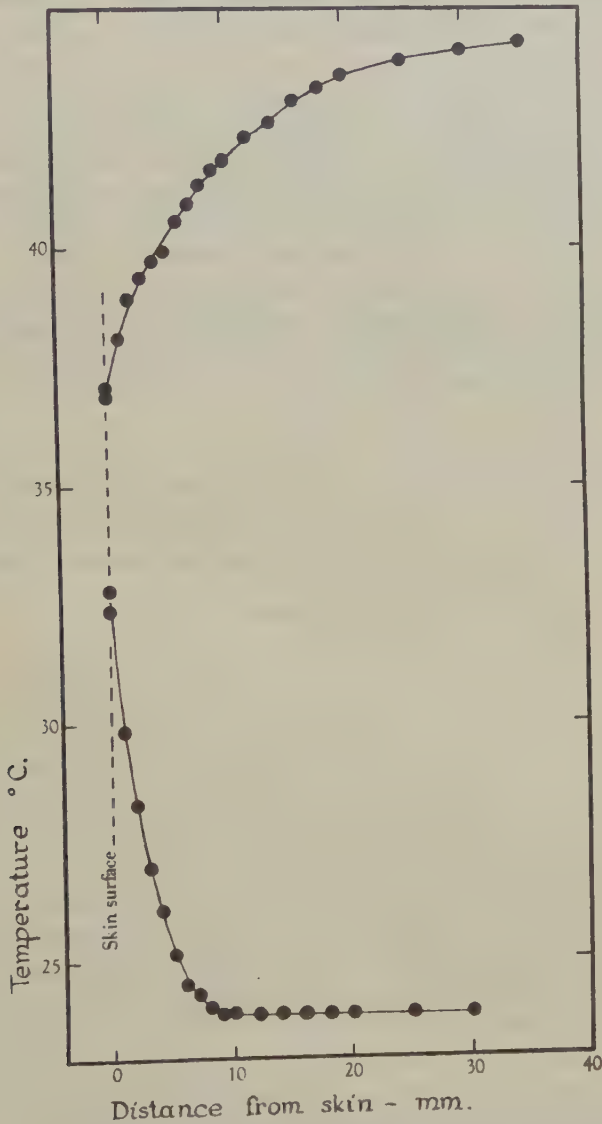


Fig. 2. Temperature gradients between the skin surface and 4 cm. distance in the experimental chamber under normal working conditions. Lower curve: environment below skin temperature; upper curve: environment above skin temperature.

clear from the present experiments, as a considerable proportion fed when the environment was warmer than the skin surface, and the highest proportion fed when environment and skin temperatures were close together. The discrepancy

between the present results and those of Bishop & Gilchrist (1946) may perhaps be explained by the alcohol-washed membranes used by these workers lacking some of the olfactory characters normal to skin. Rivnay (1931) has recorded that mouse and rabbit skin and human stratum corneum lost their attractiveness to *Cimex lectularius* after washing with soap or with ether.

THE DESTINATION OF THE BLOOD MEAL IN THE MOSQUITO

It was found in preliminary work that mosquitoes which had taken only small quantities of blood could easily be overlooked on macroscopic examination. All mosquitoes, therefore, were dissected, and the stomach and diverticula examined under the binocular dissecting microscope. It should be restated here that mosquitoes were excluded from the skin surface at the end of the feeding time by the interposition of a card, chloroformed for removal from the chamber, and dissected, usually immediately but sometimes as long as 90 min. subsequently. By visual examination the organs were placed in one of four classes; the results of this examination are shown in Table 3.

Table 3. *Distribution of blood meal to stomach and diverticula of 590 Aedes aegypti females*

Key. ++, organ fully distended with blood; +, organ partly distended with blood; tr., a trace only of blood present; —, blood absent.

	Stomach			
	++	+	tr.	—
Diverticula				
++	0	1	0	1
+	9	8	0	1
tr.	12	9	1	0
—	316	32	0	200

Of the total number of 590 mosquitoes, 200 showed no blood in any organ. Only 42, or 10.8%, of the remaining 390 mosquitoes which had taken blood showed it in any of the diverticula. It is apparent from the large preponderance of mosquitoes with blood in the stomach only that this is the usual destination of the whole blood meal in *A. aegypti*.

Marshall & Staley (1932) have summarized some of the conflicting results of previous workers regarding the function of the oesophageal diverticula in relation to blood meals, and have further carried out careful examination of this matter in British species of *Anopheles*, *Aedes*, *Theobaldia* and *Culex*. After normal feeding they found small quantities of blood in the diverticula in 16, or 9.9%, of 161 mosquitoes. Bishop & Gilchrist (1946) examined the destination of the meal in considerable detail, varying both the character of the meal and the means by which it was offered, by open drops or through membranes. They concluded that the nature of the food rather than the method of feeding determined its destination,

whether this was the stomach or diverticula of the mosquito. Only on very few occasions were blood constituents (haemoglobin in plasma or in water) found in the diverticula. The present work agrees, therefore, with the general conclusion of Marshall & Staley (1932) and of Bishop & Gilchrist (1946). Philip (1930) stated that interruption of feeding resulted in regurgitation of blood into the diverticula, and Marshall & Staley (1932) have further examined this point by chloroforming twenty-five mosquitoes in the act of biting. Four of these mosquitoes, dissected subsequently, had blood in the diverticula perceptible with low-power magnification, and the total was raised to seventeen by high-power examination. The occurrence of blood in the diverticula in the present work has been examined in relation to the temperature and humidity at which the mosquitoes were fed and the percentage

Table 4. Contingency table showing occurrence of blood in the diverticula in mosquitoes fully, and partly, distended with blood. Observed values are entered in the lower right corners of cells, theoretical values, assuming no association, in the upper left corners

		State of mosquitoes		
		Fully distended with blood	Partly distended with blood	Totals
Diverticula	With blood	37·37 31	4·63 11	42
	Without blood	309·63 316	38·37 32	348
Totals		347	43	390

mortality and degree-hour aggregates of lots. No significant correlation was found. If, however, the mosquitoes are divided into two groups depending on whether they were fully, or only partly, distended with blood, and these groups are associated with the presence or absence of blood in the diverticula, the arrangement given by Table 4 results. χ^2 for this arrangement is 11·04 and, with one degree of freedom, $P < 0·01$; the difference is significant. The conclusion of Philip (1930) and of Marshall & Staley (1932) that interruption of feeding results in regurgitation of blood to the diverticula is therefore confirmed. It is remarkable, however, that in two cases (see Table 3) a large quantity of blood was found in the diverticula, while the stomach appeared quite free from blood under the binocular dissecting microscope. It seems improbable that regurgitation from the stomach could be so complete as to leave no trace under such examination, and it is probable that the blood in these instances passed direct to the diverticula. The rarity of such an occurrence is, probably, an index of its abnormality.

THE ROUTINE FEEDING OF *AÈDES AEGYPTI* FOR
EXPERIMENTAL WORK

It is of value to summarize here the results of the present work and those of Seaton & Lumsden (1941) as far as they are important in deciding a standard technique for work in which it is desired to obtain regularly a high proportion of gorged *A. aegypti* after a single opportunity of feeding. The mosquitoes should be starved in the dark at about 24° C. and 80% relative humidity after hatching. They should be used between 72 and 120 hr. old. They should be applied to the host in a small container whose interior is in darkness and maintained at a temperature of 30–35° C. Precise control of the humidity of the air in the container seems unnecessary. An exposure time of 10 min. is ample.

SUMMARY

1. Work on the factors controlling the avidity of *Aedes aegypti* females for a blood meal previously reported by Seaton & Lumsden (1941) was continued.
2. Mosquitoes were kept after hatching for 96–120 hr. in darkness at about 25° C. and 80% relative humidity, and were then offered a blood meal on the human arm in darkness.
3. Wide variations in the light intensity during the few minutes' manipulation immediately previous to offering the host did not significantly affect the numbers feeding.
4. The relative humidity of the environment at the time of biting appeared to be of little or no importance.
5. The optimum environmental temperature for biting was near 35° C. At 35° C., out of batches of ten mosquitoes offered 5 min. opportunity to feed, means of 8.8 ± 0.44 and 9.2 ± 0.33 fed at high and low humidities respectively.
6. Large proportions of *A. aegypti* females, confined within 8.5 cm. of a host and in darkness, were able to locate the host and to feed when environmental temperatures approximated closely to the skin temperature or even were above it. Some factor other than the presence of a temperature gradient rising to the skin must therefore be responsible for their orientation to, and probing of, the host.
7. In the majority of fed mosquitoes blood was found in the stomach alone, but partly fed mosquitoes showed a higher proportion with blood in the diverticula than did those fully gorged. Such an effect is probably due to regurgitation of blood from the stomach when feeding is interrupted before its normal completion.

I am deeply indebted to Prof. P. A. Buxton, F.R.S., and to Dr Kenneth Mellanby for their interest, help and encouragement and to Messrs S. A. Smith and D. Sheffield for the preparation of apparatus and maintenance of larval cultures.

REFERENCES

- BISHOP, A. & GILCHRIST, B. M. (1944). A method for collecting sporozoites of *Plasmodium gallinaceum* by feeding infected *Aedes aegypti* through animal membranes. *Nature, Lond.*, **153**, 713.
- BISHOP, A. & GILCHRIST, B. M. (1946). Experiments upon the feeding of *Aedes aegypti* through animal membranes with a view to applying this method to the chemotherapy of malaria. *Parasitology*, **37**, 86.
- BOURNE, W. A. (1946). A resistance-type skin thermometer. *Brit. Med. J.*, **2**, 332.
- BUXTON, P. A. (1931). The measurement and control of atmospheric humidity in relation to entomological problems. *Bull. Ent. Res.*, **22**, 431.
- EDWARDS, F. W. (1941). *Mosquitoes of the Ethiopian Region. III. Culicine Adults and Pupae*. London: British Museum.
- HOWLETT, F. M. (1910). The influence of temperature on the biting of mosquitoes. *Parasitology*, **3**, 479.
- LANDOLT-BÖRNSTEIN (1923). *Physikalisch-Chemische Tabellen*, 5th ed. edited by ROTH, W. A. & SCHEEL, K. Berlin: Julius Springer.
- LARSEN, E. B. (1943). The importance of master factors for the activity of Noctuids. Studies on the activity of insects I. *Ent. medd.*, **23**, 353.
- LEWIS, D. J. (1933). Observations on *Aedes aegypti* L. (Dipt. Culic.) under controlled atmospheric conditions. *Bull. Ent. Res.*, **24**, 363.
- LUMSDEN, W. H. R. & BERTRAM, D. S. (1940). Observations on the biology of *Plasmodium gallinaceum* Brumpt, 1935, in the domestic fowl, with special reference to the production of gametocytes and their development in *Aedes aegypti* (L.). *Ann. Trop. Med. Parasit.*, **34**, 135.
- MARCHOUX, E., SALIMBENI, A. & SIMOND, P. L. (1903). La fièvre jaune. *Ann. Inst. Pasteur*, **17**, 665.
- MARSHALL, J. F. & STALEY, J. (1932). On the distribution of air in the oesophageal diverticula and intestine of mosquitoes. Its relation to emergence, feeding and hypopygial rotation. *Parasitology*, **24**, 368.
- Meteorological Observer's Handbook* (1926). London: H.M.S.O.
- PHILIP, C. B. (1930). Possibility of mechanical transmission by insects in experimental yellow fever. *Ann. Trop. Med. Parasit.*, **24**, 493.
- RIVNAY, E. (1931). Studies in tropisms of the bed bug *Cimex lectularius* L. *Parasitology*, **24**, 121.
- SEATON, D. R. & LUMSDEN, W. H. R. (1941). Observations on the effects of age, fertilization and light on biting by *Aedes aegypti* (L.) in a controlled microclimate. *Ann. Trop. Med. Parasit.*, **35**, 23.
- SIMPSON, G. G. & ROE, A. (1939). *Quantitative Zoology*. New York: McGraw-Hill Book Co.
- TATE, P. & VINCENT, M. (1935). The biology of autogenous and anautogenous races of *Culex pipiens* L. (Diptera-Culicidae). *Parasitology*, **28**, 115.
- WIGGLESWORTH, V. B. (1939). *The Principles of Insect Physiology*. London: Methuen.
- WIGGLESWORTH, V. B. & GILLET, J. D. (1934). The function of the antennae in *Rhodnius prolixus* (Hemiptera) and the mechanism of orientation to the host. *J. Exp. Biol.*, **11**, 120.

STUDIES ON TAPEWORM PHYSIOLOGY

III. ASEPTIC CULTIVATION OF LARVAL DIPHYLLOBOTHRIDAE
IN VITROBy J. D. SMYTH, *Department of Zoology, Trinity College, Dublin, Eire*

(Received 17 April 1947)

(With Plate 8 and Three Text-figures)

INTRODUCTION

The difficulty of keeping cestodes alive outside the host body for periods long enough for metabolic determinations to be carried out has been mainly responsible for the absence of detailed knowledge of the physiology of these helminths. The main problem has been a technical one, and centres on the fact that aseptic conditions are essential for successful cultivation. The starting-point in culture attempts must therefore be directed primarily towards the attainment of asepsis and parasitologists 'will have to adopt a technique more akin to that of the bacteriologist than that of the physiologist' (Wardle, 1937).

Following this line of approach, it has recently been shown (Smyth, 1946, 1947 *a*) that by commencing with the *larval* rather than the *adult* cestode phase, the pseudophyllidean cestodes *Schistocephalus solidus* and *Ligula intestinalis* can be cultured to sexual maturity *in vitro*. Since the larval stages (plerocercoids) of the above-named species occur in the coelomic cavity of fish (i.e. a natural aseptic environment)—the problem of initial microfloral contamination does not arise.

At room temperatures, plerocercoids of *Schistocephalus* have been kept alive in peptone-broth for periods up to 300 days. Adult worms of both *Ligula* and *Schistocephalus* were obtained from the plerocercoids *in vitro* by raising the temperature of cultivation to 40° C. (i.e. the body temperature of birds which form the definitive hosts for these species of cestodes). The adults so produced were apparently normal histologically, but the eggs produced *in vitro* were found to be infertile. It is evident that a similar technique can be applied to the plerocercoid larvae of any cestode found in the coelomic cavity of fish—and there are a great many of such forms known.

In the case of *Ligula* and *Schistocephalus*, the plerocercoids are already of considerable size when removed from the fish and the rudiments of the genitalia are present and well defined; the problem of inducing larvae to become sexually mature *in vitro* is therefore comparatively simple. The plerocercoids of many other cestodes, however, are very minute and never develop the *anlagen* of the genitalia while in the cold-blooded host. In such forms, considerable growth must take place before the genitalia develop and the sexually mature worms are formed. The present experiments deal with a minute larva of this type, and their objective has

been to determine whether: (a) the technique used for obtaining aseptic cultures of large forms can be successfully applied to minute forms; (b) the media in which *Ligula* and *Schistocephalus* become sexually mature are suitable for growth and development of such minute larvae.

The previous literature on culture attempts *in vitro* has recently been reviewed in detail elsewhere (Smyth, 1947*b*) and will not be given here.

MATERIAL

The larvae used were those of an unidentified pseudophyllidean cestode from the body cavity of *Gasterosteus aculeatus*. The fish were obtained from Thryberg Reservoir, near Rotherham, Yorkshire. Specimens were collected by means of traps, rod and line, and nets. About 90% of the fish examined were infected. Plerocercoids occurred both free and encysted in many sites in the body cavity of the fish—the liver being the most frequently and the most heavily infected.

It was not possible to identify the species with any degree of certainty. The size range was 1–10 mm. long and 0.5–2.0 mm. wide. The scolex was invaginated in larvae freshly removed from the fish (Pl. 8, fig. 2), but became evaginated when the organisms were relaxed in tap water at 40° C. for a few minutes. (Pl. 8, fig. 3). The relaxed and evaginated scolex (Pl. 8, fig. 1) was of the typical pseudophyllidean type with very deep lateral bothria. The outline of the scolex was triangular or oval but showed great variation depending on the degree of relaxation. Externally, the surface was rugose in appearance, but this condition was probably only superficial and it is doubtful whether any true segmentation was present.

The presence of lateral bothria places the cestode in the family Diphyllbothriidae. The above description agrees closely with that of the plerocercoids of *Diphyllbothrium ditremum* and *D. dendriticum* which have been reported from fresh-water fish in Eire (Harris & Hickey, 1945); the adults of these species occur in gulls. The reservoir at Thryberg is frequented by numerous gulls and other fish-eating birds, and it is possible that the larvae used in the present experiments may be those of one or both of the above-named species. Satisfactory identification, however, can only be based on detailed examination of sexually mature proglottids, and since these were not available for examination, it would be unwise to speculate further on possible identification without additional evidence.

TECHNIQUE FOR ASEPTIC CULTIVATION

The technique used for aseptically removing the larvae from infected fish was essentially similar to that described for *Schistocephalus* and *Ligula* (Smyth, 1946; 1947*a*) with further modifications and improvements. The specimens of fish were considerably smaller (3–4 cm. long) than those used for the work on *Schistocephalus*, and some difficulty was encountered in devising a satisfactory method for holding the fish securely during dissection. The procedure can be conveniently described in the form of instructions, which are given below:

(1) Holding the fish lightly between the fingers and taking due care not to rupture the body wall, kill it by pithing with a fine needle.

(2) Dry the fish with a soft cloth, and cut away the paired fins.

(3) Grip the skull firmly, but carefully, with a pair of large-toothed forceps (Text-fig. 1, *FL*) and clamp these forceps in a retort clamp. Grip the tail with a second (smaller) pair of forceps (Text-fig. 1, *FS*) and clamp these forceps in another clamp in an adjacent retort stand. The fish should now be firmly held in a horizontal position with the ventral surface facing the operator.

(4) Place a strong light slightly to the left of the operator and arrange it to shine on to the surface of the fish. (It is difficult to locate minute larvae deep within the body cavity unless good illumination is used.)

(5) Sterilize the surface of the skin by painting with a coat (or two if necessary) of iodine (saturated solution in absolute alcohol) and allow to dry.

(6) While the iodine is drying, remove the dissecting instruments from the sterilizer and place in a convenient position near the right hand.

(7) Make a median anterior incision in the body cavity with a fine cornea knife, and continue the cut backwards to the pelvic girdle. Cut through the pelvic girdle with fine scissors (it is almost impossible to do this with the cornea knife alone, as the strain produced is sufficient to tear apart the body from the skull). Continue the median incision with the knife to within a few millimetres of the anus. Grip the upper skin flap with a fine forceps and cut it away with the scissors.

(8) Using a flamed platinum loop in the right hand, pick up the minute larvae lying free in the body cavity and transfer to the culture tube held in the left hand. The cotton-wool plug can be conveniently removed with the little finger of the right hand, following the usual bacteriological procedure.

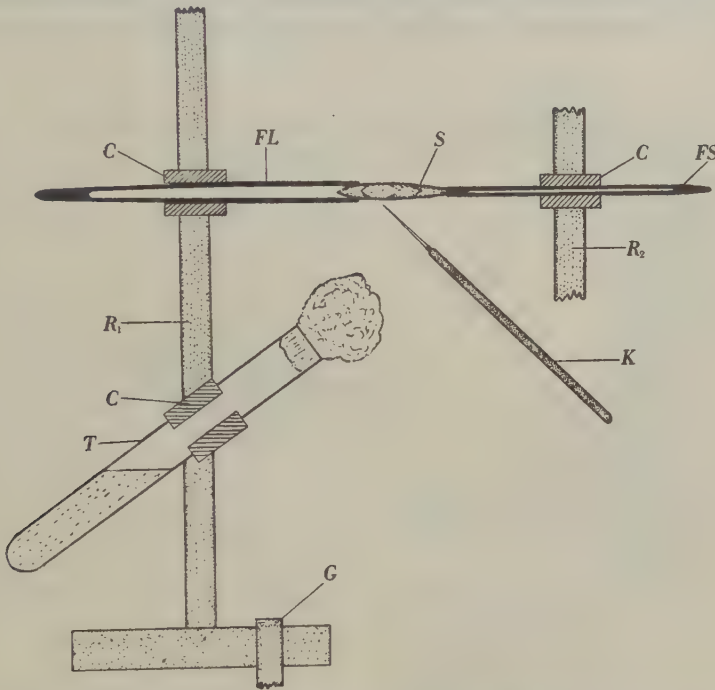
(9) For the removal of encysted larvae it is convenient to have the culture-tube clamped as shown in Text-fig. 1; both hands are thus left free. The larvae are freed by teasing away the host capsule with the cornea knife (left hand) while pressing lightly on it with the loop (right hand).

The above procedure requires some practice but is easily mastered. It is advisable to carry out the actual dissection in a room which is as free from draughts as possible, in order to reduce the possibility of contamination from air-borne spores of micro-organisms. Ideally, a sterile room is indicated. Apart from the maintenance of stringent bacteriological procedure, *the most important controlling factors in the attainment of successful aseptic cultures are rapidity of dissection and manipulation.*

Larvae were cultured in plugged rimless bacteriological culture tubes containing 10 c.c. of medium. The behaviour of the larvae was examined periodically by means of the reflecting microscope arrangement described previously for *Schistocephalus*. The present experiments were essentially directed towards the establishment of a satisfactory aseptic technique and only a small range of media was used. The basis of the nutrient media used was the commercial meat extract known as 'Lab-Lemco' (prepared for bacteriological purposes by Messrs Oxo Ltd., London) but other nutritional substances—glucose, yeast, serum—were used in addition. A 1% solution of Lab-Lemco in 0.5% sodium chloride formed the basic 'broth'. The saline media employed was $\frac{3}{4}$ Locke's saline (referred to as $\frac{3}{4}$ Locke). Previous work (Smyth, 1946) has shown that this dilution is approximately isotonic with *Schisto-*

cephalus which is also parasitic in the coelomic cavity of *Gasterosteus*; and it seemed reasonable to conclude that a similar concentration would also be isotonic with larval Diphyllbothriidae from the same host.

Culture tubes containing media were incubated at 40° C. for 48 hr. before use in order to eliminate any accidentally infected. The tubes were removed from the incubator just prior to the dissection of the fish, so that larvae commenced their period of cultivation in warm media; when the cultures were prepared they were returned to the incubator immediately.



Text-fig. 1. Arrangement of apparatus used during aseptic dissection of fish. Both retort stands are clamped securely to the bench. C, clamps; FL, large-toothed forceps; FS, small forceps; G, G-clamp; K, cornea knife; R₁, R₂, retort stands; S, fish with paired fins removed; T, culture tube.

The viability of the larvae at room temperatures was not determined in detail. Of eight larvae kept in broth + 1% peptone + 0.5% glucose at room temperatures, seven still showed active undulation on exposure to light after 80 days' cultivation.

EXPERIMENTAL RESULTS

Results of cultivation experiments are given in Table 1. Duration of normal behaviour was taken as an approximate criterion for the suitability of the culture media. 'Normal behaviour' may be defined as that period during which the larvae exhibit active movement of the scolex and strobila. The end of the period of normal behaviour was characterized, in the majority of larvae, by the loss of activity and subsequent degeneration of the scolex; in larvae dying without degeneration (most

saline media) the appearance of abnormal swelling, twisting or tetanus of the organisms was taken as indicating the end of the normal behaviour period.

In nutrient media, most larvae remained active for 2-7 days after degeneration had commenced; during this post-degeneration period, the non-degenerating region continued to undulate in a very normal manner. Larvae usually died when about half the strobila had undergone degeneration. On the average, large larvae had a longer post-degeneration period than small ones, whereas the duration of normal behaviour (as far as could be judged purely by observation) was apparently independent of the size. For this reason the duration of normal behaviour can be considered to be a more reliable (but still very approximate) criterion than the total viability.

Table 1. *Viability and duration of normal behaviour in various media at 40° C.*

Ref. no. of exp.	No. of larvae used	No. of sterile cultures	% asepsis	Medium	Viability in days			Period of normal behaviour in days				
					Min.	Max.	Mean	Min.	Max.	Mean = $\bar{M}$	S.D. of mean = σ	S.E. of mean = σ_M
38	8	5	62	$\frac{3}{4}$ Locke	2	5	4	2	4	3.4	0.9	0.4
62A	5	5	100	$\frac{3}{4}$ Locke	4	4	3	1	4	3.0	1.0	0.45
64	6	6	100	$\frac{3}{4}$ Locke	5	5	5	1	4	2.2	1.2	0.49
68	10	10	100	$\frac{3}{4}$ Locke + 0.25 % glucose	2	4	2	2	2	2.0	0	0
65	10	10	100	$\frac{3}{4}$ Locke + 0.5 % glucose	2	3	2	2	2	2.0	0	0
69	5	5	100	$\frac{3}{4}$ Locke + 1 % glucose	0	4	2	0	1	0.7	0.6	0.23
67	10	10	100	Broth	2	3	2	2	2	2.0	0	0
24B	5	5	100	Broth + 1 % peptone	5	10	9	4	6	4.5	0.8	0.33
53	10	4	40	Broth + 1 % peptone + 0.5 % glucose	9	12	11	5	6	5.5	0.7	0.36
71	6	6	100	Broth + 1 % peptone + 1.0 % glucose	7	13	10	2	4	3.3	1.0	0.40
59	10	10	100	Broth + 1 % peptone + 0.5 % glucose + 0.5 % yeast	9	9	9	3	6	4.3	1.1	0.33
62B	6	5	83	$\frac{3}{4}$ broth + 0.75 % peptone	5	14	10	2	7	5.2	1.7	0.77
40	10	9	90	Broth + 0.5 % glucose	1	6	3	1	5	3.1	1.3	0.43
30	6	4	66	Broth + 10 % horse serum	9	12	11	9	10	9.2	0.5	0.22

The duration of normal behaviour showed some variation for individual larvae even in the same media, but—excepting Exp. 69 ($\frac{3}{4}$ Locke + 1 % glucose)—the variation was small enough to allow statistical methods to be applied. Differences in the duration of normal behaviour periods in different media were considered

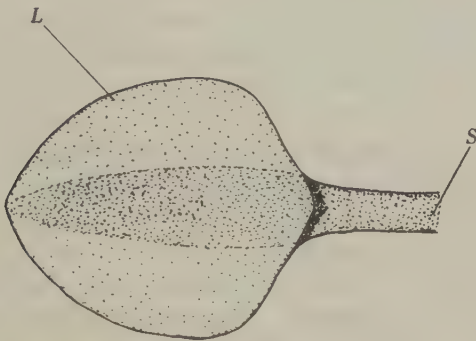
significant if $\frac{M_1 - M_2}{\sqrt{[(\sigma_{M_1})^2 + (\sigma_{M_2})^2]}}$ was greater than 2.

A detailed account of the behaviour in the various media is given below.

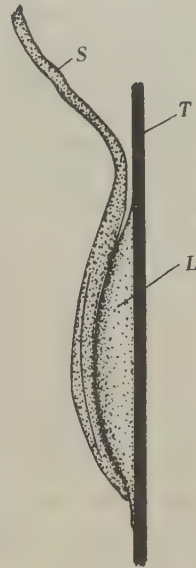
Nutrient media. Activity in pure broth was markedly sluggish, the maximum normal behaviour period being 2 days, and the maximum viability 3 days. The addition of peptone greatly increased the activity, and the behaviour figures in

peptone-broth and three-quarter strength peptone-broth (max. = 7 days) are significantly higher than those of pure broth. The addition of yeast or glucose to peptone-broth did not appreciably affect the observable activity or significantly influence the normal behaviour times. Activity and general behaviour was most marked in serum-broth, and larvae in this media showed periods of normal behaviour (9–10 days) very significantly higher than those of any other nutrient media.

The behaviour of the larvae prior to the onset of degeneration was essentially the same in all nutrient media apart from the differences in the degree of activity as noted above. Immediately on immersion in the warm media, larvae became extremely active and underwent rapid expansion and contraction. Within an hour



Text-fig. 2



Text-fig. 3

Text-figs. 2, 3. Scolex of larva, during cultivation *in vitro*, showing bothrial lips drawn out to form a large sucker firmly attached to walls of culture tube. Text-fig. 2, as seen from below, through wall of culture tube. Text-fig. 3, as seen from the side. L, bothrial lips; S, strobila; T, wall of culture tube.

the vesicular membrane along the entire length became wrinkled in appearance and was gradually shed off within the next 12 hr. The membrane was cast off first from the scolex end, and the freeing of the membrane from this end was accompanied by evagination of the invaginated scolex. The evaginated scolex showed very marked undulant movement and the movements of the bothrial lips were plainly observed. After some hours larvae became attached to the walls of the tube by their bothria—one bothrium only effecting attachment. Some larvae even became attached to strobila of other larvae in the same tube, or to their own strobila by folding backwards. When attached the lips of each bothrium became greatly expanded in a remarkable manner so that when viewed through the tube walls with the reflecting microscope, the bothrium gave the appearance of a leech-like sucker almost circular

in outline (Text-figs. 2, 3). The bothria obtained a very firm hold on the tube walls and considerable shaking was necessary to free attached larvae.

As cultivation continued the strobila became considerably lengthened and so thin that the excretory canals and even the minute calcareous corpuscles were easily discernible by reflected light. As the larvae lengthened faint cross-striations became visible; these later became more distinct, and larvae surviving for 5 days or more showed definite segmentation.

The approach of degeneration was signalled by the slowing down of undulant movement of the scolex. Within a few hours, this movement ceased completely and the scolex became opaque and limp. The degeneration then progressed from the scolex backwards until the death of the larva.

Saline media. In pure $\frac{3}{4}$ Locke, behaviour and activity for the first 1-3 days was not significantly different from that in peptone-broth, most larvae becoming attached to the walls of the tubes in the manner described above. Activity soon fell off and larvae became contracted and died rapidly—the majority of larvae showing no typical degeneration of the scolex, although posterior degeneration sometimes occurred. The addition of glucose in concentrations of 1 or 0.5% had a highly significant effect on the behaviour and viability. Activity in $\frac{3}{4}$ Locke + 0.5% glucose was considerably less than in pure $\frac{3}{4}$ Locke, few larvae became evaginated and no bothrial attachment occurred; all larvae were contracted and abnormal after 2 days' cultivation and died within 3 days. $\frac{3}{4}$ Locke + 1% glucose was toxic to the larvae and behaviour in this medium was very abnormal. All larvae showed abnormal behaviour within 24 hr.; four of the five larvae died within 48 hr. but one larva survived for 4 days. In $\frac{3}{4}$ Locke + 0.25% glucose, the activity was comparable to that in pure $\frac{3}{4}$ Locke but the behaviour figures were significantly lower and identical with those for $\frac{3}{4}$ Locke + 0.5% glucose; attachment to the tube walls never occurred although the scolex evaginated normally.

In both saline and nutrient media, no significant pH change was recorded after cultivation.

MICROSCOPICAL OBSERVATIONS

(1) *General histology*

Pieces of non-degenerating regions of the larvae were fixed after cultivation for varying times in the following media: pure broth, peptone-broth, glucose-broth, $\frac{3}{4}$ Locke, $\frac{3}{4}$ Locke + 0.5% glucose.

Histological examination revealed that no somatic growth had occurred during cultivation; no evidence of mitosis was observed in any of the nuclei in any part of the strobila. Although some larvae showed small accumulations of nuclei in the central region of the parenchyma it is questionable whether these can be taken to represent the developing *anlagen* of the genitalia. The area of transverse section of cultured larvae was considerably smaller than those of fresh uncultured forms—an effect due to the marked elongation during cultivation. The excretory system showed marked changes. In fresh larvae, the lumen of the lateral excretory canals—which lie for the most part around the periphery of the section—are small (Pl. 8,

fig. 4). In cultured larvae, the excretory canals are clearly visible as large oval or circular spaces (Pl. 8, fig. 5, *E*) which fill about one-quarter the area of the section. The two central excretory canals (Pl. 8, fig. 7, *EC*) undergo no marked change after cultivation.

(2) Distribution of fat

Material was fixed in formol-saline, imbedded in gelatine. Frozen sections were prepared and stained with Sudan Black following the usual technique. The handling and staining of frozen sections of such minute forms—even with the aid of a dissecting microscope—is one of very considerable difficulty, and sections were invariably wrinkled or slightly damaged, although the quantity of the fat present was adequately demonstrated (Pl. 8, fig. 6).

(a) *Fresh uncultured larvae*. Sections were almost *devoid of fat*. Minute droplets were occasionally seen under oil immersion. The vesicular membrane enclosing the larva stained a faint blue-black. Owing to the very minute quantity of fat present, it was found impossible to obtain microphotographs of the normal thin sections. In very thick (20μ) sections, however, the thickness enhanced the staining effect, and in addition to the vesicular membrane, the nerve cord and the central excretory vessels stain *lightly* (Pl. 8, fig. 7).

(b) *Cultured larvae*. After cultivation in all media mentioned above (Section (1)), larvae were found to contain very large quantities of fat (Pl. 8, fig. 6). Indeed, so much fat was present that it is difficult to define its exact distribution. The main quantity was present in the parenchyma and the longitudinal muscles, but under oil immersion fine droplets were found to be present over the entire section with the exception of the central excretory canals. The lateral excretory canals were for the most part fat-free (they are not clearly visible in the microphotograph); some few contained small droplets.

(3) Distribution of glycogen

Glycogen was demonstrated by means of Best's carmine or Langerhan's iodine techniques, staining being effected by the modifications described in detail by Bensley (1939). With suitable fixation and imbedding both methods gave excellent results. For microphotographs sections were stained in the iodine solution *without dissolving the wax*; by this procedure the brown-red colour of the stained glycogen appears almost black against the white colour of the wax (Pl. 8, fig. 4) and can be photographed. Stained glycogen does not photograph well in slides cleared in the usual manner.

Material for glycogen demonstration was fixed in Carnoy and imbedded *overnight* in wax after careful dehydration and clearing. Preliminary trials had shown that this prolonged imbedding was essential for uniform results. In the preliminary tests, when a number of pieces of a fresh plerocercoid were fixed and imbedded in wax for the usual times (about 2 hr.), it was found that although some blocks gave sections rich in glycogen, others gave sections very poor in glycogen—although all the material used was from the same worm! When these irregular results were investigated in detail, it was found that after prolonged imbedding, uniformly stained sections of fresh plerocercoids were *always* obtained. Moreover, material

that had been imbedded for a short time, and found to give sections poor in glycogen, when *reimbedded overnight* gave sections rich in glycogen. It would appear therefore that the dense masses of intercellular glycogen found in cestodes are very impermeable to wax, and when not properly permeated the glycogen is easily lost in the staining solutions. When properly imbedded overnight, sections can be left standing in water for periods up to about an hour without any appreciable glycogen loss; with such sections it is unnecessary to use the usual celloidin film method.

(a) *Fresh uncultured larvae*. Considerable quantities present. Distribution confined mainly to two regions—the parenchyma and the outer periphery of the longitudinal muscles (Pl. 8, fig. 4). In these regions it is present in the form of large amorphous masses which almost completely fill all the intercellular spaces. Under high power it can be seen that glycogen is present as minute granules in the region of the circular muscle fibres, and in the subcuticula.

(b) *Cultured larvae*. It is difficult to compare the quantity of glycogen present after cultivation with that in fresh uncultured larvae owing to the fact that fresh larvae are wide in transverse section, whereas cultured larvae—owing to the relaxation during cultivation—are very narrow in section. Comparisons have therefore to be made between sections of different area. This being the case, it is advisable merely to record that glycogen is present; except in cases where there has been a considerable glycogen loss a quantitative comparison is not possible.

The following cultured larvae were examined:

(i)	Broth	84 hr.
(ii)	Broth + 1 % peptone	9 days
(iii)	Broth + 0.5 % glucose	6 days
(iv)	$\frac{3}{4}$ Locke + 0.5 % glucose	84 hr.
(v)	$\frac{3}{4}$ Locke	37, 64, 84 hr.; 5 days

Sections of (i), (ii) and (iii) showed considerable quantities of glycogen still present in the *non-degenerating* tissue regions. The degenerating scolex region and the regions immediately behind this were quite free from glycogen. (iv) was completely glycogen-free. In (v) quantities of glycogen were present after 37 and 64 hr. cultivation, but completely absent after 84 hr. and 5 days.

DISCUSSION

These experiments can be regarded as successful as far as the attainment of aseptic cultures is concerned, but unsuccessful as regards further growth and development of the plerocercoid beyond the primitive larval condition. The technique of aseptic cultivation has now been applied to the whole size range of plerocercoids: the largest known larval form, *Ligula intestinalis*, with a maximum size of about $\frac{1}{2}$ m.; an intermediate sized form, *Schistocephalus solidus*, with a maximum size of about 6 cm.; and the minute larvae—with a size range as given above—used in the present experiments. Since a great many pseudophyllidean cestodes have their plerocercoid phase in the coelomic cavity of fish, it seems likely that in time the aseptic cultivation technique will be successfully used on a number of these forms. The

fact that location in the coelomic cavity of fish determines that larvae are living in an aseptic environment does not seem to have been generally realized.

With reference to the behaviour during cultivation, the strong attachment of the scolex to the wall of the culture tube gives some indication of the powerful muscular action of the bothria. The degree of development of the bothria in cestodes can thus be correlated approximately with the duration of life within the definitive host. The form of scolex in the larvae used in the present experiments is very similar to that of *Diphyllbothrium latum* and it is reasonable to conclude that in this latter form the scolex must become firmly attached to the intestinal wall of the host. Once attached, the cestode could undergo considerable growth, and in the case of *D. latum* there is a record of its persistence in man for a period of 8 years (Leiper, 1936).

In both *Schistocephalus* and *Ligula*, on the other hand, the bothria are very feebly developed—they are little more than shallow grooves. Attachment of these forms to the culture tube *in vitro* has never been observed, from which it is reasonable to conclude that a firm hold is never obtained on the gut wall *in vivo*. It can be speculated then, that these worms can only remain in the host gut for a short time. This is, in fact, exactly the condition found—the worms pass through the bird gut within 48 hr. (*Schistocephalus*) and 72–96 hr. (*Ligula*). The longer period of the latter is probably accounted for by the fact that the larger size of worm would find its passage down a coiled gut more difficult.

These few preliminary experiments, carried out as they were with a limited range of media, provide little additional information as to the nutritional requirements of cestodes. The available evidence, reviewed elsewhere (Smyth, 1947*b*) suggests that glucose, vitamins, hormones and an unidentified factor present in autoclaved yeast are necessary for normal growth and development. In the present experiments the variation between the normal behaviour periods between larvae in different media was statistically significant only in some cases.

Broth alone gave poor results, but the addition of 1% peptone was definitely beneficial. The further addition of glucose or yeast to the peptone-broth did not give significantly better results. The addition of 10% horse serum to the peptone-broth resulted in a marked improvement both in the normal behaviour period and the observable activity. This result suggests that the natural body fluids are likely to provide a culture medium more suitable for growth and development than one synthesized from simple inorganic and organic compounds.

The experiments with the saline solutions show that such solutions are useless for cultivation work, although results with these media are of interest. Behaviour was best in pure $\frac{3}{4}$ Locke's solution. The addition of 1 or 0.5% glucose was definitely detrimental to the normal behaviour. This result is interesting, for in the early experiments on *Schistocephalus* (Smyth, 1946) it was suspected that the addition of glucose to saline media had a detrimental effect on behaviour, although the results were not sufficiently uniform to allow a statistically significant result to be obtained. It is curious that glucose exerts such an effect on larvae in saline solutions, for the addition of 1% glucose to peptone-broth had no apparent effect—either beneficial or otherwise.

All the little available evidence on the carbohydrate metabolism suggests that glucose can readily be absorbed by cestodes and stored in the form of glycogen. Markov (1939) found that when the plerocercoids of *Diphyllobothrium latum* were kept in Locke's solution at 15° C. for 38 hr., the glycogen content fell to 66% of its initial level; for the same period at 35° C. the glycogen content fell to 22%. After 72 hr. at 35° C., the glycogen content fell to 6%. In Ringer-Locke + 1% glucose after 38 hr. cultivation at 35° C. a gain of 3% in the glycogen content was recorded. These results are possibly open to criticism on the grounds that the experiments were not conducted under sterile conditions; for in non-sterile media, behaviour is very abnormal and degeneration rapidly sets in. Since glycogen is a notably labile substance and rapidly disappears from dead or degenerating tissue, the glycogen losses may have been due to this cause, although the fact that a gain was recorded in glucose-Locke after 38 hr. would suggest that the tissues remain normal for at least this period. However, the fact that non-sterile cultures were used introduces an uncertain factor into Markov's work, and his results—though of great interest—must be accepted with caution. Reid (1942) carried out feeding experiments on chickens and found that the glycogen content of the cestode *Raillietina cesticillus* fell to one-eleventh of its initial level after 20 hr. starvation of the chicken host.

In the present experiments, the results of histochemical analysis of glycogen carried out on larvae after cultivation in various media, while not at variance with Markov's results, are so inconclusive and uncertain that they provide little additional data on the carbohydrate metabolism.

The fact that in pure broth (i.e. a glucose-free medium) considerable quantities of glycogen are still present after 84 hr. cultivation suggests that either the cestode can synthesize glycogen from some of the complex substances* present in the meat extract or that the glycogen loss is not as considerable in this form as the results of Markov and Reid would seem to suggest. The low viability in pure broth did not allow glycogen determinations to be made after a more prolonged period. It is thus not possible to draw any conclusions from the presence of considerable quantities of glycogen in larvae after cultivation in broth + peptone (9 days), or broth + glucose (6 days). The cestode may be able to synthesize glycogen from peptone as well as from glucose, but there is no evidence that this is so.

Under starvation conditions, i.e. in $\frac{3}{4}$ Locke's solution, the complete disappearance of glycogen from larvae cultured for 84 hr. or longer would seem to support the hypothesis that the glycogen reserves in cestodes are very rapidly used up under starvation conditions. It is doubtful, however, whether such a conclusion is justifiable on this evidence alone, for considerable quantities of glycogen were present after 64 hr. cultivation, and it is difficult to believe that very little glycogen was used during the first 64 hr. of cultivation and that all the glycogen was suddenly consumed during the following 20 hr. A more likely explanation is that after about 72 hr. the worms were slowly dying and the disappearance of the glycogen was due to this cause. The results in $\frac{3}{4}$ Locke + 0.5% glucose would seem to confirm this

* Albumose, gelatine, peptones.

latter conclusion. If the larvae had been quite normal when fixed, and glucose was being absorbed, the tissues would have been rich in glycogen. Histochemical examination, however, revealed that all the glycogen had disappeared after 84 hr. cultivation. It seems wisest therefore to conclude that behaviour in salines or glucose-salines is abnormal and for this reason it is not justifiable to draw any conclusions as to the carbohydrate metabolism.

In contrast with glycogen, the increase in fat in sections of cultured larvae as compared with fresh larvae was so very striking and consistent that it can be concluded, with certainty, that fat is produced during cultivation. The fact that fat is a by-product of cestode metabolism has long been suspected but definite proof has been lacking; previous evidence has been summarized elsewhere (Smyth, 1947*b*). Brand (1933) suggested that the fat is formed probably by the anaerobic breakdown of glycogen. In both *Ligula* and *Schistocephalus* a similar production of fat has recently been demonstrated (Smyth, 1947*c*) and in these latter forms there is a marked excretion of fatty acids into the culture medium and the pH falls very rapidly. The fact that no pH drop was recorded in the present experiments is accountable when the very minute size of the larvae is considered. As pointed out already, the results from the histochemical glycogen investigations were very indefinite and it cannot be concluded that this fat is produced at the expense of the glycogen. Further investigations of the glycogen-fat relationship must await detailed chemical analysis.

The results of the present experiments have shown that a simple medium such as peptone-broth, in which large plerocercoids of *Ligula* and *Schistocephalus* (i.e. those containing genitalia *anlagen*) can become sexually mature, is not suitable for the growth and development of minute larvae. The problem of the *in vitro* cultivation of these small forms can therefore be considered to be in a position similar to that of animal and plant tissue culture after the early pioneer experiments had been carried out, namely, the basic technique of aseptic cultivation has been established, but a suitable medium in which growth and development will take place had not yet been elaborated. The elaboration of such a growth-producing medium must necessarily be a matter of empirical research. In addition to the composition of such a medium, physico-chemical factors—pH, osmotic pressure and temperature—must also be investigated in detail.

SUMMARY

1. Unidentified plerocercoids of the family Diphyllbothriidae were removed aseptically from the coelomic cavity of *Gasterosteus aculeatus* and cultivated at 40° C. in various liquid nutrient and saline media under sterile conditions.

2. The most successful results were obtained with peptone-broth + 10% horse serum in which larvae remained active and behaved normally for 10 days. Broth without peptone gave poor results (max. 2 days) but the addition of peptone (max. 7 days) greatly improved the activity. The addition of glucose or yeast to peptone-broth was not significantly beneficial. Survival times in all nutrient media were considerably longer than the periods of normal behaviour.

3. In $\frac{3}{4}$ strength Locke's saline, the maximum period of normal behaviour was 4 days. The addition of glucose (0.25–1%) to this saline was detrimental to the behaviour of the larvae.

4. Histochemical investigation showed that fresh larvae were rich in glycogen but almost fat-free. The occurrence of glycogen in larvae after cultivation in various nutrient and non-nutrient media was such that no conclusions regarding the carbohydrate metabolism could be drawn. All larvae after cultivation, however, contained *great quantities* of intracellular fat, from which it was concluded that fat is a by-product of metabolism.

5. During cultivation in the more favourable media, larvae became firmly attached to the walls of the culture tube by means of their bothria; the strobila also became greatly elongated, and segmented in some cases. Histological examination failed to detect mitosis in the nuclei of the strobila and it was concluded that growth of the strobila had not taken place. The elongation of the strobila was presumed to be a result of the relaxation of the longitudinal musculature.

6. Of eight larvae kept in broth + 1% peptone + 0.5% glucose at room temperatures, seven still showed active undulation on exposure to light after 80 days' cultivation.

REFERENCES

- BENSLEY, C. (1939). *Stain Tech.* **14**, 47.
 BRAND, T. VON (1933). *Z. verg. Physiol.* **18**, 562.
 HARRIS, J. R. & HICKEY, M. D. (1945). *Nature, Lond.*, **156**, 447.
 LEIPER, R. T. (1936). *J. Helminth.* **14**, 127.
 MARKOV, G. S. (1939). *C.R. Acad. Sci. U.R.S.S.* **25**, 93.
 REID, W. M. (1942). *J. Parasit.* **28**, 319.
 SMYTH, J. D. (1946). *J. Exp. Biol.* **23**, 47.
 SMYTH, J. D. (1947*a*). *Parasitology* **38**, 173.
 SMYTH, J. D. (1947*b*). *Biol. Rev.* **22**, 214.
 SMYTH, J. D. (1947*c*). Unpublished work.
 WARDLE, R. A. (1937). *Canad. J. Res. D*, **15**, 117.

EXPLANATION OF PLATE 8

Fig. 1. Enlargement of evaginated scolex shown in fig. 3. Gilson; borax carmine. $\times 110$.

Fig. 2. Entire plerocercoid fixed immediately after removal from fish. Scolex invaginated. Gilson; borax carmine. $\times 30$.

Fig. 3. Entire plerocercoid after relaxation in tap water. Scolex evaginated. Gilson; borax carmine. $\times 10$.

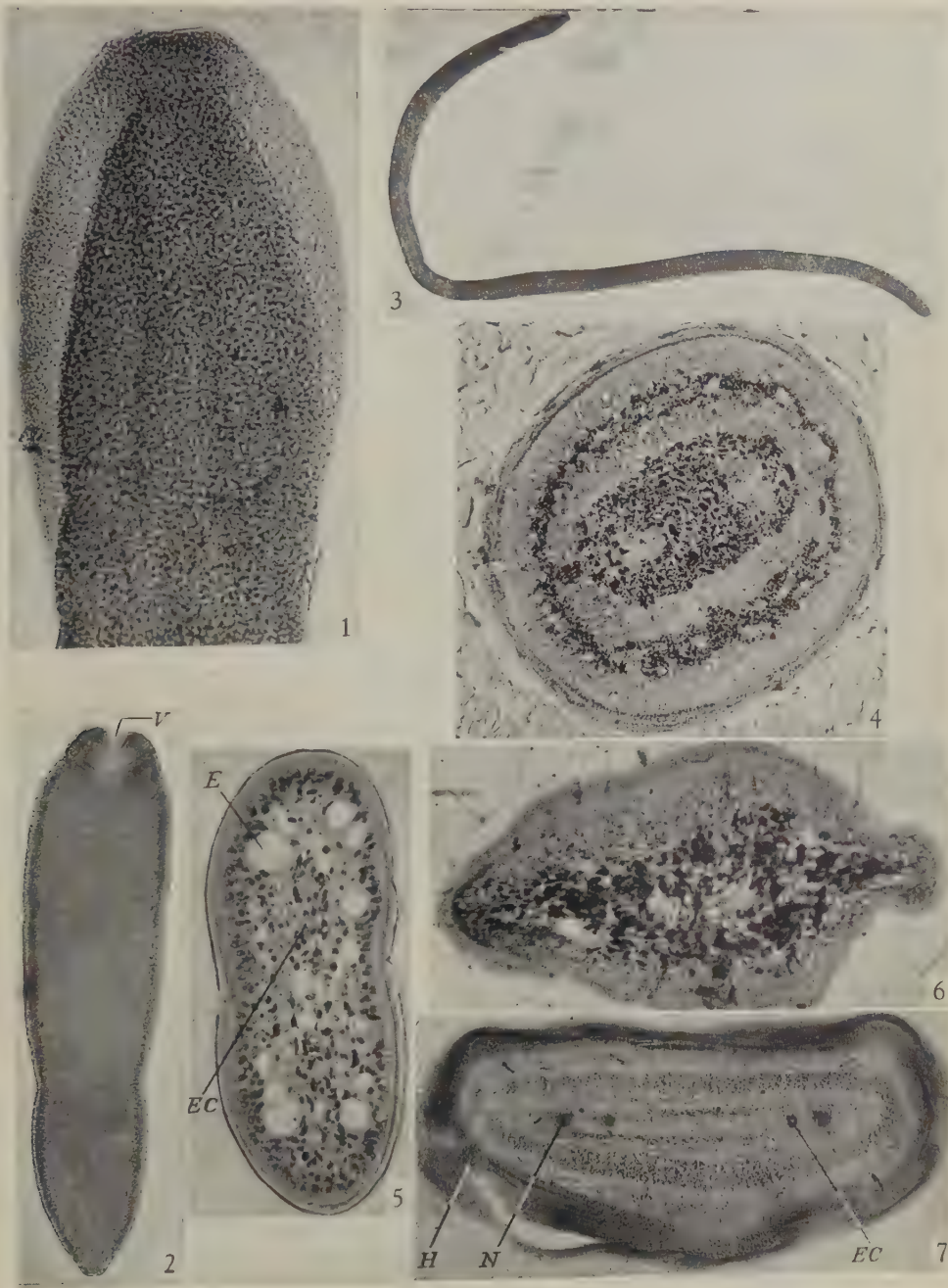
Fig. 4. Transverse section of uncultured plerocercoid showing distribution of glycogen confined mainly to parenchyma and muscular regions. Section stained and photographed without removal of wax. Carnoy; Lugol's iodine. $\times 100$. (This microphotograph has been considerably touched up; staining of glycogen is intense but does not photograph too well.)

Fig. 5. Transverse section of plerocercoid after cultivation for 5 days in peptone-broth. Note great enlargement of lateral excretory canals. Bouin. Haematoxylin and erythrosin.

Fig. 6. Transverse section of plerocercoid after cultivation for 9 days in peptone-broth. Great quantities of fat present. Formol-saline; gelatine imbedded; Sudan Black.

Fig. 7. Transverse section of fresh, uncultured plerocercoid; very thick section (20μ). Very little fat present, and only found in vesicular membrane (*H*), nerve cord (*N*) and central excretory canals (*EC*). Parenchyma and muscle layers fat-free. Compare with fig. 6. Formol-saline; gelatine imbedded; Sudan Black.

Abbreviations: *E*, lateral excretory canals; *EC*, central excretory canals; *H*, vesicular membrane enclosing larva; *N*, nerve cord; *V*, invaginated scolex.



SMYTH—STUDIES ON TAPEWORM PHYSIOLOGY

AN ELECTRO-MAGNETIC MIXER FOR MANOMETRIC EXPERIMENTS

BY H. LASER AND LORD ROTHSCHILD

From the Molteno Institute and Department of Zoology, Cambridge

(Received 2 June 1947)

(With Three Text-figures)

INTRODUCTION

Manometric experiments often involve the addition of substances such as a substrate, an inhibitor, or acid, to the material under investigation. This is usually done in the following ways:

By running the substance into the main vessel from a turnable side bulb.

By tipping it in from a fixed side bulb.

By dislodging a dangling cup or 'Keilin tube' within the manometer vessel.

These methods have disadvantages in certain types of experiment. In all three the shaker has to be stopped when the substance is added, while in the last two, the vessel must be removed from the bath. Furthermore, in the first method, the substance cannot be added quantitatively and the turning of the side bulb may not always leave the manometer reading unaffected. These disadvantages are usually unimportant, as in ordinary experiments the gas change is of a fairly large order, and extrapolation can cover the first few minutes after the addition of the substances. The disadvantages involved in these methods are, however, accentuated if readings have to be made at short intervals after the addition of the substance (at intervals of less than 1 min. for example), and especially if the Warburg indirect method is used, when substances have to be added to both vessels at the same time.

These difficulties arose while investigating the fertilization reaction in the eggs of the sea urchin, *Psammechinus miliaris* (Laser & Rothschild, 1939), when the Warburg indirect method was used and very short interval readings were required after adding the spermatozoa to the egg suspension.

A method has therefore been developed for adding substances to the manometer vessels of a differential manometer without stopping the shaker or removing the vessels from the bath. The principle consists in holding a Keilin tube in the top of the manometer vessel by means of an electrically maintained magnetic field. The tube, which contains the substance to be added, in this case the sperm suspension, is allowed to drop into the egg suspension by interrupting the electric current. This device enables substances to be added simultaneously, or at known intervals, to any number of vessels. The method has the further advantage that small main vessels can be used, as the Keilin tube can be suspended in the hollow part of the manometer stopper. Consequently, the volume of the main vessel need not be enlarged

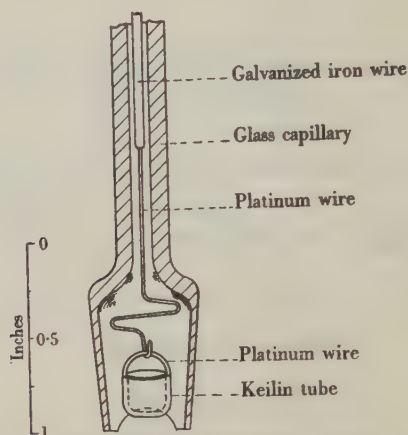
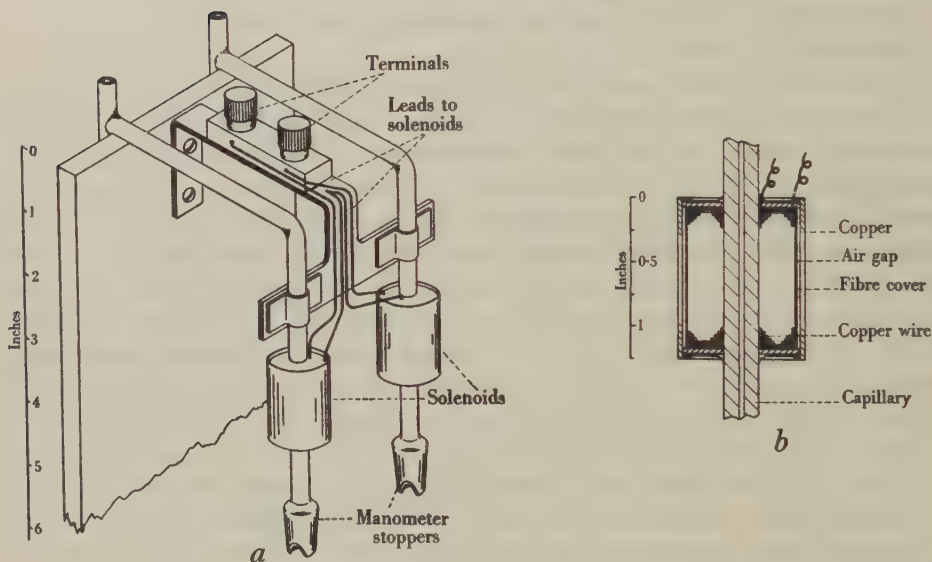
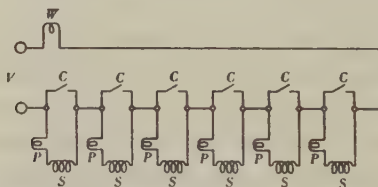


Fig. 1. Manometer stopper, with Keilin tube in position.

Fig. 2. *a*, rear view of manometer, showing solenoids on capillaries above manometer stoppers. *b*, section of solenoid.Fig. 3. Complete wiring diagram for six mixers. *V*, 110 V. d.c. supply; *W*, 60 W. bulb; *C*, switches; *P*, pilot lamps; *S*, solenoids.

by the volume of the side bulb, which would reduce the sensitivity of the apparatus. If two substances have to be added consecutively, two side bulbs are necessary (Dixon, 1943, p. 95) and this also is obviated by the electromagnetic mixer.

TECHNICAL DETAILS

The Keilin tube is hooked on to a piece of platinum wire in the hollow part of the manometer stopper (Fig. 1). At its distal end the platinum wire is connected to a length of galvanized iron wire, which is held by a magnetic field in the capillary tubing above the stopper. The manometer stoppers are cut away to provide a clear opening between the gas phase in the manometer and the capillary, as shown in Fig. 1. The Keilin tube is hooked into position and the magnetic field switched on before the vessel is put on to the manometer.

The magnetic field is produced by coils of wire or solenoids round the outside of the capillary tubing above *both* manometer vessels, and as the wattage is the same in each there is no asymmetrical heating effect. Each solenoid contains 1000 turns of silk covered no. 28 copper wire, and their total resistance is about 7 ohms. Constructional details of the solenoids are shown in Fig. 2*b*. Resin flux and not acid flux should be used to prevent corrosion of the copper wire.

The lengths of the iron and platinum wires holding the Keilin tube are adjusted so that when the magnetic field is switched off only the platinum wire comes into contact with the liquid in the manometer vessel.

The complete wiring diagram is shown in Fig. 3. It is convenient to have a pilot light associated with each coil in the circuit, and these should light up whenever a coil is energized. Neither the number of turns of wire in each solenoid, nor the gauge of the wire, nor the external voltage are critical.

We are much indebted to Mr M. J. Hubbard for making the electrical apparatus.

REFERENCES

- DIXON, M. (1943). *Manometric Methods*. Cambridge.
LASER, H. & ROTHSCHILD, LORD (1939). *Proc. Roy. Soc. B*, **126**, 539.

RHYTHMICAL IMPEDANCE CHANGES IN THE EGG OF THE TROUT. III

By LORD ROTHSCILD, *From the Department of Zoology, Cambridge*

(Received 1 June 1947)

(With Plates 9-12 and Ten Text-figures)

INTRODUCTION

When a trout egg is placed in a conductivity cell in an alternating current bridge, rhythmical changes of the system's impedance are observed (Hubbard & Rothschild, 1939). These changes are reversibly inhibited by phenyl urethane (Rothschild, 1940). Analysis of the impedance cycle into its resistive and capacitive components shows that it is mainly due to changes in the capacitance of the system (Rothschild, 1947*b*). Similar changes occur in fertilized and unfertilized salmon eggs (Rothschild, 1947*a*). Few measurements have been made on the capacitances of living cells, and in particular on their plasma membranes, while data on changes in capacitance are even scarcer. The most important are:

(i) On fertilization in *Arbacia* and *Hippoonö esculentus* eggs, when the plasma membrane capacitance increases by more than 100% (Cole, 1938).

(ii) When *H. esculentus* eggs swell in hypotonic sea water, the plasma membrane capacitance decreases (Cole, 1935).

(iii) During the passage of an action potential along *Nitella* the capacitance decreases by 15% (Cole & Curtis, 1938).

(iv) During the passage of an action potential along the squid giant axon the capacitance is said by Cole & Curtis (1939) to decrease by 2%.

(v) When the mammalian erythrocyte undergoes haemolysis by water or chemical lysins there are changes in the frequency-dependent properties of the membrane capacitance (Fricke & Curtis, 1934).

(vi) According to Cole (1928, p. 53), 'In conversation Dr Hugo Fricke said that he had found a change in the capacity of a frog egg upon fertilization. . . '.

The stability of the capacitance under varying environmental conditions and during cellular reactivity has contributed to the concept of the plasma membrane as a thin, inert, and electrically insulating lipid matrix; these properties may be responsible for the large capacitance ($1 \mu\text{F.cm.}^{-2}$) that is usually observed. It is, therefore, important to consider the changes in membrane structure, or in the system as a whole, which might be responsible for the observed rhythmical changes in capacitance. The interpretation of these phenomena is made more difficult by the discovery, described in this paper, that both unfertilized and fertilized eggs undergo rhythmical movements (Pl. 9). Apart from any detailed interpretation, the

existence of these changes gives rise to a number of questions, of which the following are important:

- (1) On what metabolic system or systems are the changes dependent?
- (2) Why do the changes stop a certain number of days after fertilization?
- (3) Is it possible that the changes are not spontaneous, but are due to the treatment of the egg during measurements?
- (4) What variations in the external environment have an effect on the changes?
- (5) If these effects are peculiar to cells which are morphologically similar to the trout or salmon egg, what morphological peculiarity is responsible?
- (6) Do the changes merely indicate that the egg, or some parts of it, are in an abnormal or moribund condition?

Answers to some of these questions will be found in this paper, though it is obvious that the problems raised by these rhythmical changes cover so wide a field that complete or detailed explanations are unlikely to be forthcoming for some considerable time. The relatively short period during which trout eggs are available each year makes a general survey desirable, so that those lines of inquiry which are likely to be most profitable can be established. The following experiments have been performed and will be dealt with in this paper:

- (a) The effect of temperature changes on frequency.
- (b) Examination of the variation in frequency with age of eggs, and of the variations in frequency between eggs from the same and from different females.
- (c) The effects of sodium cyanide, sodium azide, phlorizin, sodium fluoride, chloretone, distilled water, and calcium chloride. The effects of chloretone and CaCl_2 were examined for reasons which will be discussed later. The effects of NaCN and NaN_3 in inhibiting the cytochrome oxidase system, and of phlorizin or NaF in inhibiting glycolysis, are well known. The experiments with inhibitors represent the beginning of an attempt to identify the metabolic system or systems on which the changes depend. This is a complicated problem, and one which requires specialized biochemical attention.
- (d) The effect of varying the plane of measurements relative to the egg, to see if there is any correlation between the form and frequency of the change and the morphological characteristics of the egg.
- (e) Examination of the possibility of there being a resistance change as well as a capacitance change in the system, and of the phase relationship of these two parameters.

(f) Photographs and cine-photographs of unfertilized trout eggs have been taken. I am much indebted to Mr K. Williamson for assistance in this work.

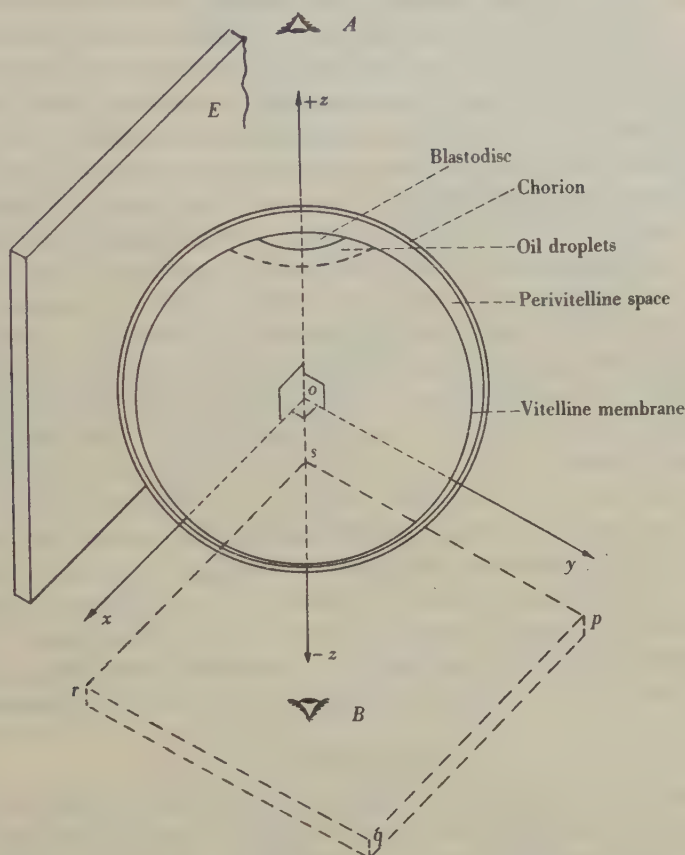
Before describing the experiments, it is advisable to consider what physical changes in the egg as a whole might be responsible for the observed impedance changes. The first of these is a periodic change in the membrane dielectric constant, which would be measured as a change of capacitance. The second possibility is a periodic change in egg volume, which is the subject of experiments described in this paper. If the egg swells, the surface membrane must become thinner, unless the egg synthesizes 'membrane material' in such a way as to keep the membrane thickness

constant, which is improbable. If the membrane becomes thinner, the capacitance will increase, and therefore if the egg swells and shrinks rhythmically, the capacitance will vary in a rhythmical manner. The simplest explanation of periodic changes in volume would be that the egg takes in and expels water periodically; but this raises difficulties because Gray's experiments (1932) show that the surface of the trout egg is virtually impermeable to water. A third possibility is that the egg changes its shape periodically. The electrical properties of a suspension of spheres are different from those of a suspension of ellipsoids, and it is therefore conceivable, though unlikely, that a periodic change in shape might account for the observed impedance changes. The last possibility is that different parts of the egg surface have different dielectric constants or capacitances, and that the position of these parts relative to the measuring system is not constant. It has long been known that fertilized eggs of certain fresh-water fish exhibit movements. These seem first to have been observed by Rusconi (1840), who thought the movements were due to cilia, and quotes W. Sharpey as saying in his article on cilia in R. B. Todd's *Cyclopaedia of Anatomy and Physiology* (1836, p. 606) that this is the case. I have not been able to find any statement to this effect in the article. Ransom (1867) made a systematic investigation of fish-egg movements, but was unable to find any movements in the trout egg. In recent years, fish-egg movements have been investigated by Yamamoto (1940) and to a lesser extent by Kuhl (1939). Detailed references to earlier papers on this subject will be found in the bibliographies at the ends of these two papers. Although movements have on rare occasions been observed in unfertilized fish eggs (Wintrebert, Painlevé & Yung Ko-Ching, 1929), no systematic experiments have been carried out on them, and there has been a tendency to associate such movements with developmental processes. In his most recent paper, Yamamoto (1940, p. 75) says: 'There are a host of observations to indicate that the eggs of animals reveal irregular amoeboid movement shortly after fertilization', while in a paper on the movements of goldfish eggs (Yamamoto, 1934), it is stated that the movements start at the four-cell stage, and that the variations in these movements with temperature are similar to those observed during developmental processes. Needham (1942, p. 75), referring to these and other movements in embryos, says: 'We are still far from any understanding of their meaning, but it will be remembered that protoplasmic contractility plays a large part in echinoderm fertilization....'

Unfertilized trout eggs undoubtedly undergo periodic movements, and it is probably due to their relatively low frequency, which is about one complete cycle every 3 min. at room temperature, that they have not been observed during experiments on these eggs in recent years. In fact, if one looks at a trout egg under a microscope in the ordinary way, no changes are noticeable, and it is only by special methods of observation, accelerated films, or by impedance records, that these changes become apparent. Such movements make the interpretation of the rhythmical impedance changes very much more difficult.

Experiments *a-c*, described above, and their interpretation, apply equally well to periodic egg movements or periodic egg impedance changes. The extent to which

the electrical changes are in part due to structural alterations in the egg surface, or to changes in egg volume, or entirely to the movements of the egg as a whole, is examined in detail in this paper; but, as is mentioned above, impedance measurements on moving systems are exceptionally difficult to interpret.



Text-fig. 1. Diagram of a trout egg, not to scale. *A*, observer looking in the negative *z* direction; *B*, observer looking in the positive *z* direction; *E*, one electrode; *O*, origin of rectangular co-ordinates; *pqr* is a plane parallel to the *x-y* plane.

MATERIAL

Unfertilized eggs of the brown trout (*Salmo fario*), the rainbow trout (*Salmo irideus*), and the *shasta* variety were used. There seemed to be no significant electrical differences between these three types of egg. Some eggs were obtained by the usual method in the laboratory; others were sent by post from the Midland Fishery in test-tubes containing about fifty eggs in spring or tap water.

Fertilized eggs were not used this season because no difference between the impedance cycle in fertilized and unfertilized eggs has been detected. It is often taken as a criterion of health if the eggs can be fertilized at the end of the experiment. This is impossible in the case of trout eggs for well-known morphological

reasons. Moreover, the moment a trout egg becomes unhealthy, the normal impermeability of the vitelline membrane disappears and salts diffuse out of the egg interior. The electrical method of measurement (q.v.) is ideally suited to following this process. Normally, the experimenter can tell when a trout egg is unhealthy or moribund, because white patches, caused by the precipitation of intracellular globulin, can be seen through the chorion, the patches corresponding to areas of permeability in the vitelline membrane. But unhealthiness, characterized by the exosmosis of salts, can be observed much earlier by electrical than by visual means, since the slightest variation in the electrical conductivity of the external medium, quite apart from changes in membrane resistance, is perceptible in an a.c. bridge.

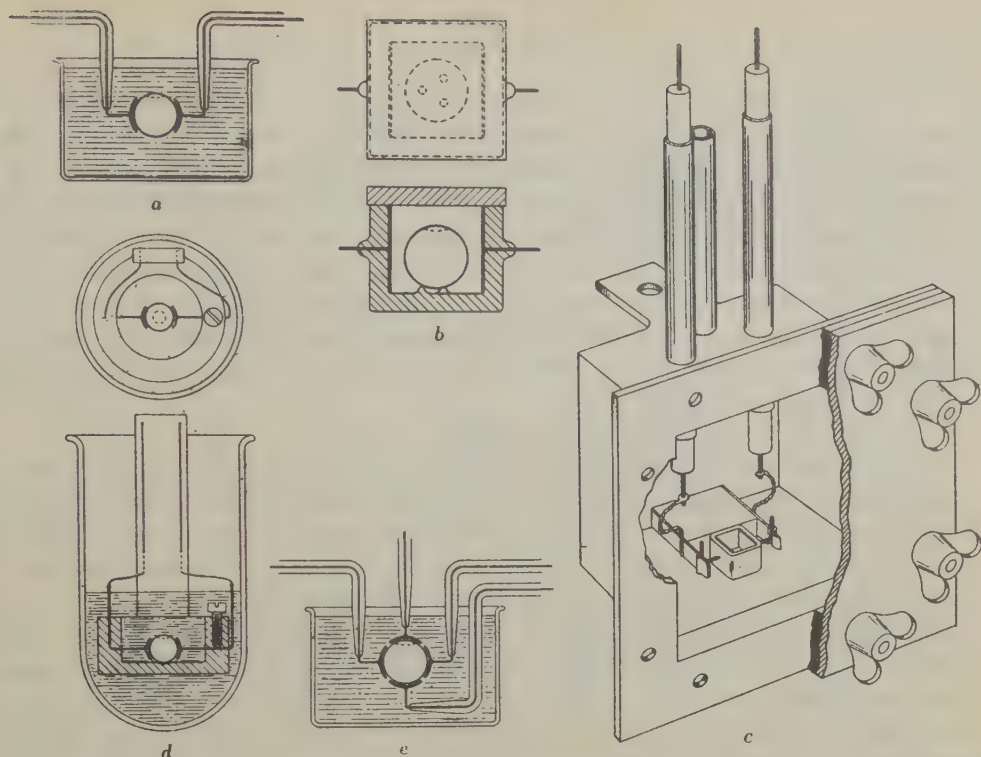
A geometrically idealized diagram of a trout egg is shown in Text-fig. 1. For purposes of subsequent discussion, three axes at right angles to each other, with their origin at the centre of the egg, are included in the diagram. By the usual convention, the observer at *A* is looking in the negative *x* direction, while the observer at *B* is looking in the positive *x* direction. The slab *E*, on the left side of the egg, is one of the electrodes, and is parallel to the *x-z* plane, but at right angles to the *x-y* and *y-z* planes. Electrodes in this position are described as being in the 'normal' position; electrodes parallel to the plane *pqrs* are described as being in the 'vertical' position.

If the egg in Text-fig. 1 is rotated through 180° , so that the blastodisc is at the south pole, the whole egg will rotate within the chorion until the blastodisc is again at the north pole, because the cap of oil droplets round the blastodisc, and possibly the blastodisc itself, make the animal pole the lightest part of the egg. The actual position of these oil droplets is not quite clear. According to Gray (1932), they are within the vitelline membrane and blastodisc, but it seems possible that they adhere fairly firmly to the insides (or undersides) of these two structures.

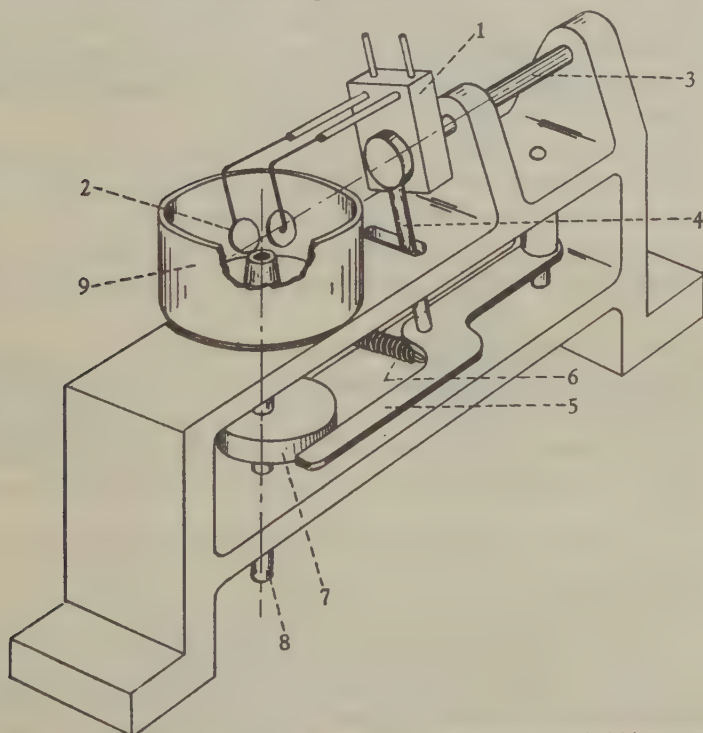
APPARATUS

Measurements were made with an a.c. bridge of conventional design. The bridge was energized by a heterodyne beat oscillator with continuously variable frequency up to 50 kcyc./sec. The detector was an oscilloscope and tuned amplifier. For some experiments the output of the oscillator was split into two parts; one part supplied the bridge and the other was applied to the *x* plates of the oscilloscope. The output from the bridge was applied to the *y* plates, and the phase relationship was so arranged that the resultant Lissajous figure was an ellipse which tilted for standard arm resistance unbalance and widened for capacitance unbalance.

Various types of conductivity cell were used and are shown in Text-fig. 2. The first of these, *a*, consists of two platinized platinum circular shell electrodes, attached to Zeiss micromanipulators, and dipping into any suitable container. This is used for preliminary experiments. The second type, *b*, is a cubical glass container, two opposite faces of which are internally covered with platinized platinum plates, the electrodes. A ground-glass plate fits on the top of the container. The base has three small conical glass supports in the centre, on which the egg sits. This cell was thermostatted, and the way this was done is shown in Text-fig. 2*c*. A third type



Text-fig. 2. Conductivity cells described in text. A trout egg, radius 0.23 cm., is shown in position in each case. Thermostatting arrangements are shown in *c* and *d*.



Text-fig. 3. 1, perspex block; 2, electrodes; 3, spindle; 4, connecting rod held in contact with lever 5, by the spring 6, which also holds 5 against the eccentric cam 7; 8, spindle carrying glass vessel 9. Rotation of 8 rotates the egg between the electrodes and rocks the electrodes on the spindle 3.

of conductivity cell is shown in Text-fig. 2*d*. It is a thermostatted version of cell *a*. Text-fig. 2*e* shows the electrode arrangement when the impedance changes are measured in different planes. Only opposite pairs of electrodes are placed on the egg surface during an experiment. A special conductivity cell is shown in Text-fig. 3. In it the base, upon which the egg sits, can be moved about in the x - y plane, relative to the electrodes, and at the same time the electrodes, which are fixed relative to each other, can be rotated round the egg. The movement of the egg in the x - y plane, and that of the electrodes, are controlled from the same drive, so that these two movements, in different planes, have a fixed phase relationship. The object of this device will be explained later.

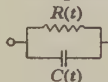
ANALYSIS

The bridge unbalance wave-form. The existing method of recording the impedance change has the disadvantage that the frequency of the actual impedance variation is *half* the frequency of the maximum unbalance values in the record. This is shown in Pl. 10*a*, which is a record of the bridge unbalance. The actual impedance change derived from this record is shown by the dotted line in Pl. 10*a*.

This effect is shown diagrammatically in Text-fig. 4, in which it will be seen that when values of R_p and C_p in the bridge standard arm are such as to balance the egg in its conductivity cell at some arbitrarily selected time during the impedance cycle, the resulting record has double the 'frequency' of the actual change; furthermore, according to the moment in time at which balance occurs, the shape of the impedance cycle appears to vary, though in fact it does not. This disadvantage in the method of recording changes would be obviated by taking records entirely outside the region of bridge balance, the impedance changes being studied unilaterally in the envelope curve; but the technical problems associated with this method of measurement make it preferable to adhere to the original method.

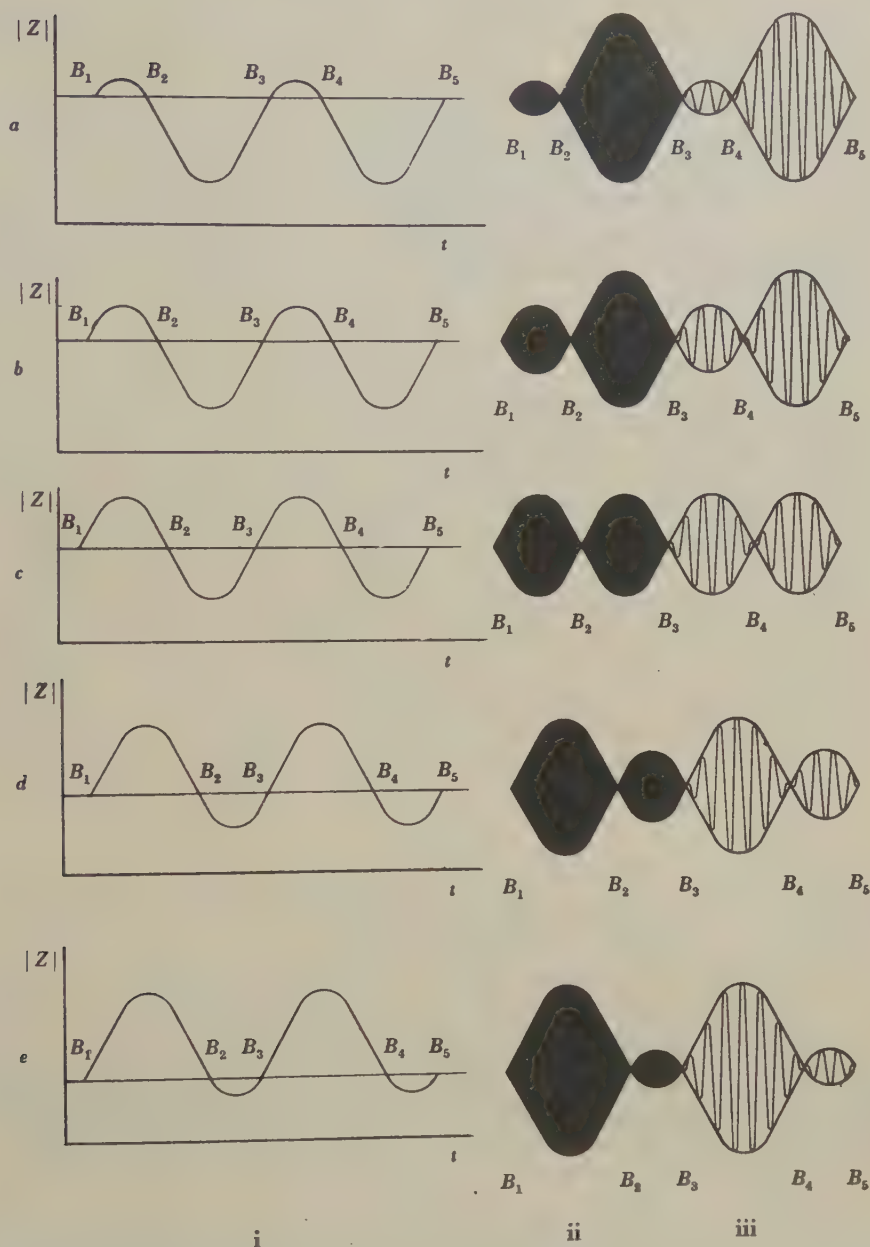
Apart from this we are not interested so much in the recorded impedance cycle as in the electrical changes in the system which produce this impedance cycle. These may be different from the observed bridge unbalance records, and it is upon the former that it is necessary to concentrate. The method of deducing how the various electrical parameters which make up the impedance vary is outlined in the following section.

Fine structure of the impedance cycle. The relationships between changes in some parts of the system comprising the unknown arm of the bridge and the equivalent changes in the bridge standard arm are somewhat complicated. They are easier to understand if one considers a much simpler network than that to which an egg in tap water is equivalent. Suppose that the unknown arm of the bridge contains the

network , and that by means of some device the values of R and C are made to vary sinusoidally, i.e.

$$R = \bar{R} + R_m \sin \Omega t, \quad C = \bar{C} + C_m \sin (\Omega t + \theta). \quad (1)$$

We use the letter Ω instead of the more normal ω for the angular frequency, so as not to cause confusion between the frequency of the variation in R and C and the



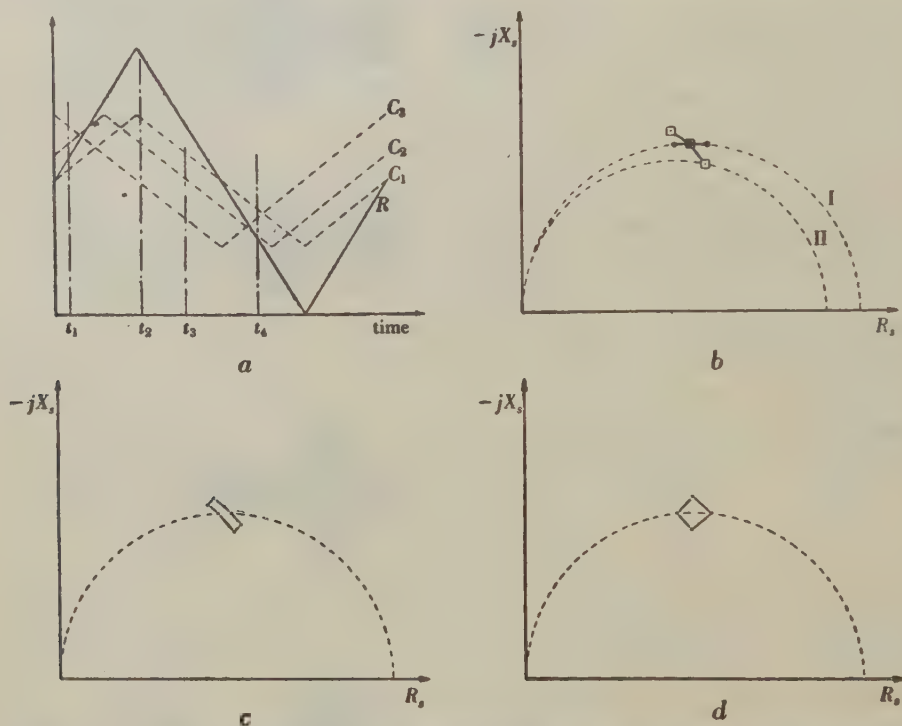
frequency of the oscillator energizing the a.c. bridge. θ is the phase difference between R and C ; it may be zero. The impedance of this varying system, at any frequency ω , will be

$$|Z| = \{\bar{R} + R_m \sin \Omega t\} \{1 + \omega^2 [\bar{C} + C_m \sin \Omega t + \theta]^2 [\bar{R} + R_m \sin \Omega t]^2\}^{-\frac{1}{2}}, \quad (2)$$

while the phase angle across the terminals will be

$$\psi = \arctan \omega \{\bar{R} + R_m \sin \Omega t\} \{\bar{C} + C_m \sin (\Omega t + \theta)\}. \quad (3)$$

Neither $|Z|$ nor ψ is necessarily sinusoidal, so a particular shape of observed impedance variation does not automatically mean that variations in the unknown



Text-fig. 5. *a*. Variation of resistance, R , and capacitance C with time. C_1 is in phase with R while C_2 and C_3 are $\frac{1}{2}\pi$ and $\frac{1}{4}\pi$ out of phase respectively. *b*. Semicircle I: $\odot$, variation of capacitance alone; $\square$, simultaneous in phase variation of resistance and capacitance. Semicircle II, change in impedance locus due to resistance change alone. *c*. Simultaneous variation of resistance and capacitance, $\frac{1}{2}\pi$ out of phase. *d*. Simultaneous variation of resistance and capacitance, $\frac{1}{4}\pi$ out of phase.

arm components are of the same form. Even if the capacitance alone changes in the unknown arm, the resulting impedance change of the network need not be sinusoidal. Consequently, the shape of the impedance change, which is measured across the terminals of the complete network in the unknown arm, provides no direct information about the form of the variations in individual components in the unknown arm.

In the very simple case examined above, the phase relationship between R and C

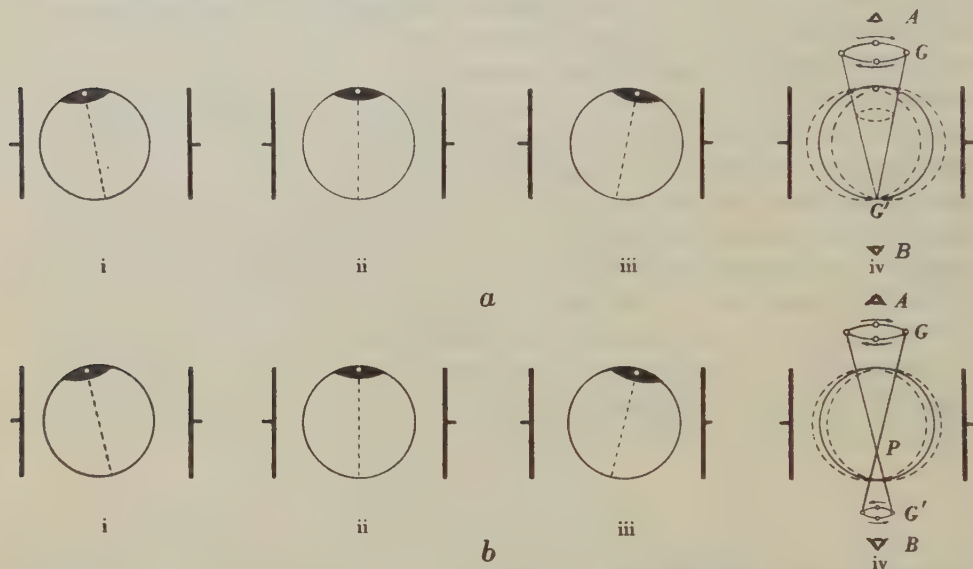
will be exactly reproduced by R_p and C_p in the bridge standard arm, because the networks in the standard and unknown arms are identical. But if the values of R_p and C_p are used to plot the impedance locus, which involves converting R_p and C_p into their equivalent series resistances and reactances at each frequency, the relative phases and wave forms of R_s and C_s (from which the reactance $1/\omega C_s$ is derived) may differ from those in the original network. There is, however, a method of establishing whether or not variations in the components in the unknown arm are in phase. Suppose that the unknown arm does consist of a resistance R and capacitance C in parallel, and that the values of R and C vary as in Text-fig. 5*a*. There is no point at this stage in making R and C vary sinusoidally, since according to their values, R_s and $1/\omega C_s$ may contain harmonics. We shall consider four conditions: R and C are in phase, C leads R by $\frac{1}{2}\pi$, C leads R by $\frac{1}{2}\pi$, R does not vary, but C does. The last condition determines the arc of the circle on which the static values of R and C lie, and is shown in Text-fig. 5*b*I. The other conditions are shown in Text-fig. 5*b*II, *c* and *d*. From these diagrams it will be clear that if the parameters in the unknown arm are not in phase, the criterion of this condition is that the time locus of the impedance (or admittance) vector terminal at the selected frequency will enclose part of the $R_s X_s$ plane and will not be a line. That this must be so is obvious from Text-fig. 5*a*; the ordinates at t_1 and t_2 cut the C_2 curve at equal C_2 values, but R is different in each case. The same applies to t_3 and t_4 , so that in all cases where the phase difference between C and R is greater than 0 and less than π , the impedance or admittance vector will trace a loop and not a line with time. The width of the figure traced out is a function of the phase difference between C and R for given values of C and R .

RESULTS

'Precession'. Accelerated cine-photographs show that the unfertilized trout egg undergoes rhythmical movements within the chorion (Pl. 9). This movement can loosely be described as a periodic axial perturbation, or wobble, about a particular point on, or in, the egg. This point may be at the south pole of the egg, or within the egg, on the line joining the north and south poles. The movement is somewhat similar to that part of the motion of a top which is known as precession. The analysis of the motion of a precessing sphere is rather difficult, both conceptually and mathematically, and a detailed consideration of this phenomenon is unnecessary here. The simplest way of considering this motion is by means of the idealized egg diagrams in Text-fig. 6. In these diagrams, the orbits of a spot on the egg surface (in reality an oil droplet) is delineated, while the egg is examined in different planes. To obtain a complete picture of the motion of a precessing sphere by visual means, an observer must look at the egg, or take accelerated cine-photographs of it, in at least three different directions.

Although it is desirable to express these periodic movements of the trout egg in an unambiguous or formal way, so as to avoid words like 'wobble', this paper is concerned with the physiological properties and electrical concomitants of these movements. Formal analysis will therefore be dealt with elsewhere.

Correlation between egg movements and impedance changes. The time taken for an oil droplet to describe one complete orbit is identical with the duration of one complete impedance pattern. The meaning of 'complete impedance pattern' is explained in the next section, entitled 'Wave-form of the impedance change'. Whenever an egg exhibits rhythmical movements, there are associated rhythmical changes in the impedance of the system; the converse is difficult to prove. The electrical method of measurement is so much more sensitive and accurate than visual examination that it is hard to establish the presence or absence of egg movements when the electrical changes have been reduced in amplitude and frequency by special treatment. We cannot as yet say that the impedance changes are



Text-fig. 6. *a.* Precession of the egg about the south pole; three positions, i, ii and iii, are shown; iv, conical surface generated by the north-south axis. The observer at *A* sees the globule *G* describing an elliptical path; the observer at *B* sees a stationary globule *G'*. In iv, the three positions i, ii, iii, of the egg show the enveloping surface over which *G* travels. *b.* Precession of the egg about the point *P* on the north-south axis. Note that in this case, the observer at *B* sees the globule *G* describing an ellipse, in the opposite sense to that seen by the observer at *A*.

exclusively the *result* of the movements, or that they are not the result, but a concomitant, of the movements, as in the case of muscle. This question is discussed later in this paper.

Wave-form of the impedance change. Examination of Pl. 10 *a* shows that every fourth bridge unbalance has a different amplitude from the previous three. In other words, the impedance cycle has an amplitude modulation every other cycle. The time interval between two consecutive modulations is the 'complete impedance pattern' referred to earlier on; the time interval is identical with the frequency of the egg movement.

This modulation has been further investigated by examining the relationship between the impedance change measured with the electrodes in the normal (lateral)

position, and with the electrodes in the vertical position (Text-fig. 2e). The results of these measurements are shown in Pl. 10b.

It will be seen that whereas the impedance change in the lateral plane consists of a 'fundamental' change modulated every other cycle, the change in the vertical plane, though in fact of the same frequency, could be described as a 'fundamental' change of half the frequency of the lateral change, with a harmonic of double the frequency.

The way the changes in the two planes are described is largely a matter of choice, because the frequency of the complete impedance pattern is the same in both cases, and the difference between them can be interpreted.

Spontaneity. The evidence that the changes in the impedance of the system are neither caused nor affected by the measuring current is:

(a) If the measuring current makes the impedance of the egg or of the whole system vary rhythmically, or alters any existing rhythmical changes in the egg, one would not expect the impedance variation to continue its normal course during an interruption of the measuring current. Pl. 10c shows the effect of interrupting the measuring current at various times and for various durations. When the current is switched on after an interruption, the impedance variation picks up exactly where it should do if unaffected by the measuring current; it behaves, in fact, as if the current had never been switched off.

(b) There is an almost linear relationship between the amplitude of the impedance variation and the voltage across the unknown arm of the bridge. The maximum and minimum values of R_p and C_p remain reasonably constant when the voltage across the unknown arm is varied (Table 1), showing that in any particular egg the

Table 1. 17 January 1947. UF egg, 3 days old, $\nu = 3000$.
Maximum and minimum values of R_p and C_p

Input voltage 25 mV.		Input voltage 50 mV.		Input voltage 100 mV.		Input voltage 200 mV.	
R_p, Ω	$C_p, 10^{-8} \text{ F.}$	R_p, Ω	$C_p, 10^{-8} \text{ F.}$	R_p, Ω	$C_p, 10^{-8} \text{ F.}$	R_p, Ω	$C_p, 10^{-8} \text{ F.}$
1027	1'343	1027	1'342	1028	1'339	1027	1'331
1004	1'332	1003	1'329	1004	1'324	1007	1'322
1027	1'346	1027	1'343	1028	1'340	1028	1'332
1004	1'331	1003	1'331	1005	1'322	1006	1'318

amplitude of the impedance variation depends only on the impressed voltage. The lack of discontinuities in the relationship between impressed voltage and the values of R_p and C_p supports the conclusion that the current energizing the bridge is not responsible for, nor has any effect on, the observed changes. The fact that fertilized and unfertilized eggs do not undergo these changes when first put into water suggests that they may be caused by the abrupt change of environment when the egg leaves the female and enters a medium with entirely different properties, though we shall see later that the comparative lack of Ca^{++} in the external medium does not seem to be the responsible factor. When therefore it is said that the effect is

spontaneous, all that is meant is that these rhythmical changes are neither produced nor affected by the measuring apparatus.

The effect of changes in temperature. The frequency of the impedance pattern is fairly constant in eggs from one female (Table 2), though eggs from different females show a good deal of variation (Table 3). It would therefore be convenient to measure a number of Q_{10} 's on eggs from one female; but this is hard to do, because of the time each temperature run takes, and the difficulty of doing more than one or two

Table 2. 29 January 1947. *Impedance pattern frequencies in unfertilized eggs from the same female trout. $T^{\circ}\text{C. } 16.8$*

Egg no.	Frequency (min.)	Egg no.	Frequency (min.)
1	3.30	7	3.40
2	3.40	8	3.32
3	3.35	9	3.74
4	3.30	10	4.08
5	3.36	11	3.32
6	3.44	12	3.24

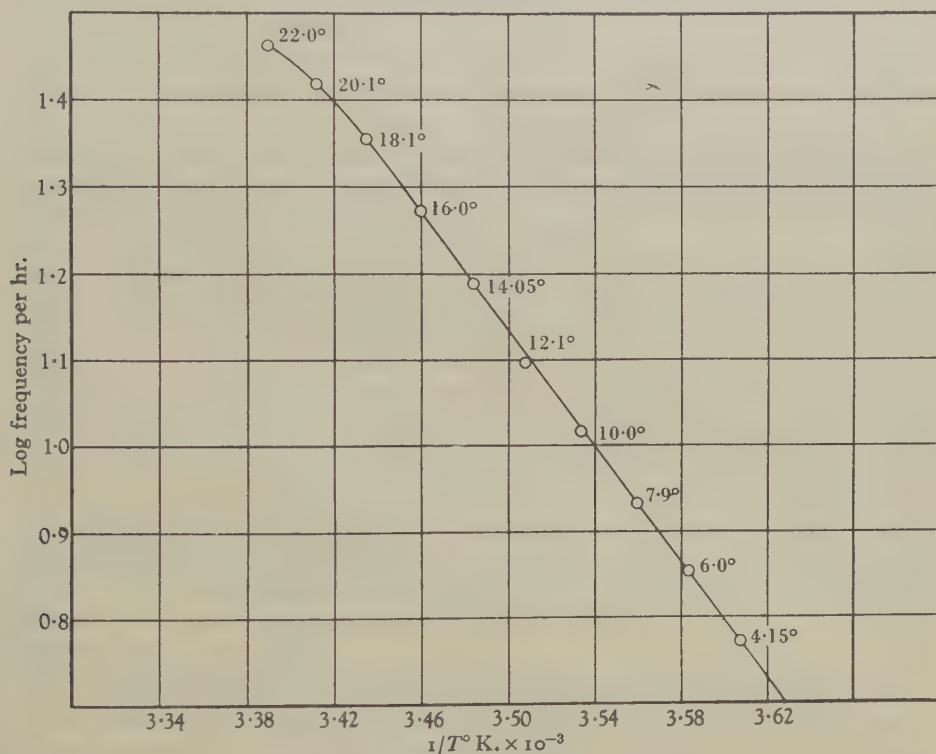
Table 3. *Impedance pattern frequencies in unfertilized eggs from different female trout*

Egg no.	$T^{\circ}\text{C.}$	Frequency (min.)	Egg no.	$T^{\circ}\text{C.}$	Frequency (min.)
20	16.0	3.46	28	16.3	4.14
21	16.0	2.92	29	16.5	4.68
22	16.1	3.47	30	16.5	4.10
23	16.1	3.41	31	16.8	4.00
24	16.1	3.44	32	16.8	5.40
25	16.1	3.56	33	16.8	5.30
26	16.1	3.41	34	16.8	4.10
27	16.1	3.42			

runs at the same time. A typical graph of the variation in frequency with temperature is shown in Text-fig. 7, while in Text-fig. 8 six separate runs are recorded, to show the extent of the variation between different eggs. In both cases the logarithm of the frequency per hour is plotted against the reciprocal of the absolute temperature; it will be seen that the Arrhenius equation is reasonably well satisfied from the lowest temperatures to about 18°C. Above 18°C. there is a marked flattening of the curve, or 'break', though the behaviour of the eggs at these higher temperatures suggests that the change in the slope of the Arrhenius plot is more likely to be a sign of irreversible injury than of a 'critical point' (Crozier, 1924*b*). At temperatures above 18°C. eggs behaved in an erratic manner. A number died during the experiments, and only a few runs were done at these higher temperatures. In Text-fig. 7 the average value of the temperature characteristic μ , between 4.15 and 18.1°C. , is 15,200, calculated from the Arrhenius equation, which corresponds to a Q_{10} of 2.47. The slight spread at the lower temperatures, which is visible in Text-fig. 8, is real; it reflects the fact that the rhythmical changes are affected by these low temperatures, and not always in a reversible manner. The

mean value of the temperature characteristic for seventeen runs (54 measurements of μ), from *c.* 18 to 5° C., was 16,200.

The effect of sodium cyanide and sodium azide. M/300, M/500 and M/1000 solutions of NaCN in tap water, with pH adjusted to that of ordinary tap water (*c.* 7.4), were used. KCN was avoided because of the well-known action of K⁺ ions on membrane resistance (Cole & Marmont, 1942; Hodgkin & Huxley, 1946). Care has to be taken in setting up and interpreting experiments to test the effect, or lack of effect, of chemical inhibitors on the impedance cycle. The addition of NaCN to tap water



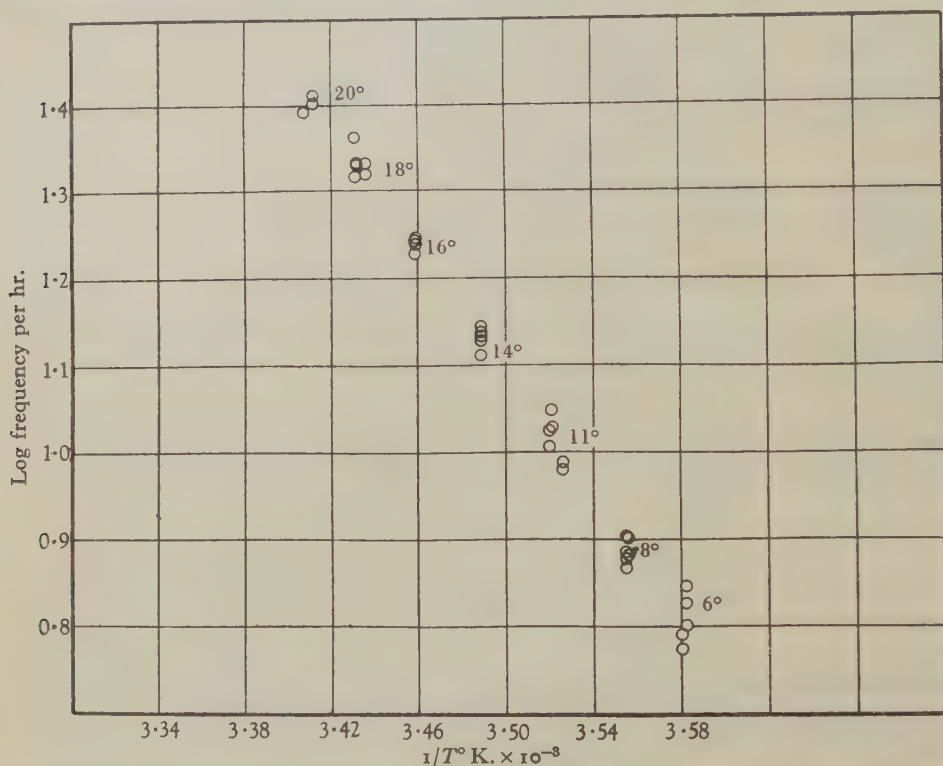
Text-fig. 7. Variation in frequency of impedance change with temperature. Ordinates, log frequency; abscissae, reciprocal of absolute temperature. The temperatures against each co-ordinate are in ° C.

alters its conductivity, and control experiments must therefore be made in tap water with conductivity no lower than that of the test solution. Moreover, it is obviously essential that the control tap water should not have any effect on the impedance cycle. This latter point presented difficulties in the case of the controls for the experiments with tap water containing NaN₃ (q.v.), but not in the case of the cyanide experiments. In the latter, the control solution was tap water to which NaCl was added until the conductivity was about the same as that of the pH-adjusted, NaCN-tap water solution. NaCl has no effect on the impedance cycle, at any rate in the low concentrations needed for these purposes.

Experiments to determine the effect of inhibitors on the electrical properties of

a single cell are difficult to compare with conventional manometric experiments in which, for example, the actual percentage of cyanide-sensitive respiration can be determined. In these experiments there are four possible effects of NaCN which may be expected: reversible reduction in amplitude, reversible alteration in frequency, reversible abolition, irreversible abolition.

To attempt a more strictly quantitative investigation would, at this stage, be unprofitable. It must be remembered that though a trout egg is the size of a small pea, the amount of actual metabolizing tissue present is exceedingly small.



Text-fig. 8. Six temperature runs to show individual egg variations. Ordinates, log frequency; abscissae, reciprocal of absolute temperature. Approximate temperatures in °C.

M/1000-NaCN reversibly reduces the frequency of the oscillation. Pl. 11 shows this effect, the egg beating at $\frac{1}{3}$ cycle/min. before treatment; it also shows various phases in the reduction of frequency during immersion in M/1000-NaCN tap water, and the egg recovering its normal frequency, when replaced in its normal environment. These experiments also show that the frequency of the oscillation is much more sensitive to cyanide than is the amplitude, which barely alters even when the frequency is markedly reduced. Stronger solutions of NaCN are needed to reduce the amplitude or to abolish the oscillation altogether; even if M/1000-NaCN would eventually achieve this result, there are several factors which make it difficult to continue this type of experiment for long periods of time. The oscillation can be

abolished reversibly by $M/300$ -NaCN, but the experiments are difficult because this concentration tends to kill the egg and it is not easy to take an egg out of the cyanide solution *after* the oscillation has become invisible but *before* the egg is irreversibly damaged. This has only been achieved once or twice, though by washing in tap water one can get eggs to recover after a large reduction in amplitude through treatment with $M/300$ -NaCN solutions.

Suppose that NaCN lowers the membrane resistance and causes salts to diffuse out of the egg interior into the external medium. The impedance change, which it will be remembered is mainly capacitative, would then be shunted by a reduced resistance. Can the reduction in amplitude be accounted for by such changes in resistance, the oscillation not, in fact, being cyanide-sensitive at all? As regards the decrease in amplitude of the oscillation, this possibility cannot be absolutely discarded without quantitative measurements which present difficulties. All that can be said is that this interpretation is improbable. In any case, the *frequency*, as well as the amplitude, is affected by NaCN, and a reduction in the values of the electrical parameters can have no effect on frequency.

It would be natural to extend these results by experiments with NaN_3 instead of NaCN. Some preliminary experiments of this type have already been reported (Rothschild, 1940) and a more detailed examination has now been made. NaN_3 only exerts its effect on the cytochrome oxidase system at pH 6.6 or lower (Keilin, 1936). If the effect of NaN_3 in tap water at pH 6.5 is to be investigated, the controls must also be studied at pH 6.5. When an egg is transferred from ordinary tap water to tap water whose pH has been reduced to 6.5 with HCl, the oscillation changes in a remarkable way. This is illustrated in Pl. 10*d*. The amplitude and frequency are reduced almost instantaneously to a very low value. Recovery as regards amplitude gradually sets in, but the frequency seems to be affected for a longer time. This effect of pH reduction raises certain interesting questions, but the azide experiments at this pH necessarily had to be abandoned, as, without controls, they would have been meaningless.

The thermostating of trout eggs during electrical measurements is most easily done in a conductivity cell of the type shown in Text-fig. 2*d*, which is open at the top. Some preliminary experiments in this cell gave deceptive and unreproducible results owing to the volatility of HCN, which is the compound responsible for the effects described above. Below pH 8 almost all the cyanide is present in the form of HCN. All experiments were therefore carried out in the sealed cell (Text-fig. 2*b*) after preliminary experiments with open cells were found to be quite inconclusive. The use of sealed conductivity cells delays temperature equilibration to a certain extent, so that it is difficult to take records of the impedance cycle immediately after an egg has been put into the test solution; but in the case of the relatively weak cyanide solutions the effect is sufficiently gradual to make this delay unimportant. There are indications that stronger cyanide solutions, and possibly even weak ones, have an immediate effect on the electrical properties of the vitelline membrane. A systematic investigation of this effect can only be carried out with eggs which are not beating. According to Gray (1932, p. 289), however, $M/100$ -KCN

'has relatively little effect on the stability of the eggs if the latter are absolutely fresh and not subjected to mechanical disturbance'. It is evident that a further examination of the effect of KCN and NaCN on eggs which are not beating is necessary.

The effect of phlorizin. Experiments were carried out with phlorizin to see if the changes were in any way dependent on the phosphorylation stage of anaerobic glycolysis. M/100, M/200 and M/500 solutions of phlorizin in tap water (without pH adjustment) were used. These solutions had no effect on the impedance cycle, and unfertilized eggs left for 6 days in M/500-phlorizin in tap water continued oscillating without any signs of being affected by this solution. It can therefore be concluded either that the changes are not associated with phosphorolysis, or that the phlorizin molecule does not penetrate.

The effect of sodium fluoride. Eggs were left in M/25-NaF in tap water for 3 hr.; this treatment had no effect on the rhythmical changes. Eggs which were left in this solution for 16 hr. died though control eggs were quite healthy, and this may be compared with the results with phlorizin. Though the short-period experiments with fluoride and phlorizin may be comparable, this is not necessarily the case with the longer period experiments, as the egg batches were different. The NaF experiments were done much later in the season, when trout eggs are more delicate than earlier on.

The effect of calcium chloride. Though there are several reasons for thinking that the trout egg impedance cycle does not belong to the same class of phenomena as the action potential, it was thought advisable to see if an increase in the Ca^{++} concentration round the egg had any inhibiting effect. Lack of calcium in the external medium induces spontaneous rhythmical discharges in squid nerves (Arvanitaki, 1939). These spontaneous and rhythmical potential changes are abolished by restoring the normal Ca^{++} concentration in the external medium. The student of fertilization in fresh-water animals can hardly fail to appreciate the marked change of environment to which an egg is subjected when laid. Within the female trout, the egg is in contact with a fluid in which Δ is about -0.48°C. , while after being laid, the egg is in an environment in which Δ is about -0.02°C. , and the Ca^{++} concentration is 0.002M. The sudden change when the egg passes from one medium to the other, might, by analogy with nerve, cause rhythmical changes in the vitelline membrane.

The experiments to find out if the restoration of Ca^{++} in the external medium has any inhibitory effect on the impedance cycle were carried out with M/25- CaCl_2 in tap water round the egg, instead of the usual tap water. The conductivity controls were carried out as described in the previous section on the effect of NaCN. The experiments showed that M/25- CaCl_2 in tap water has no effect on the rhythmical changes.

The effect of distilled water. The eggs were placed in a special perfusion conductivity cell in which laboratory-distilled water was continuously led in at the bottom and siphoned off at the top. This system ensures that the egg is in contact with distilled water; but because of the effect of the chorion in hindering diffusion some

time must elapse before the vitelline membrane is in actual contact with the new medium. Since, however, the conductivity of the system varies continually while diffusion of distilled water into the perivitelline space and diffusion of tap water out into the external medium is going on, it is easy to tell when the egg is in a pure distilled water environment. Distilled water has no effect on the rhythmical changes; an egg was left in it for 48 hr. and was unaffected by this treatment.

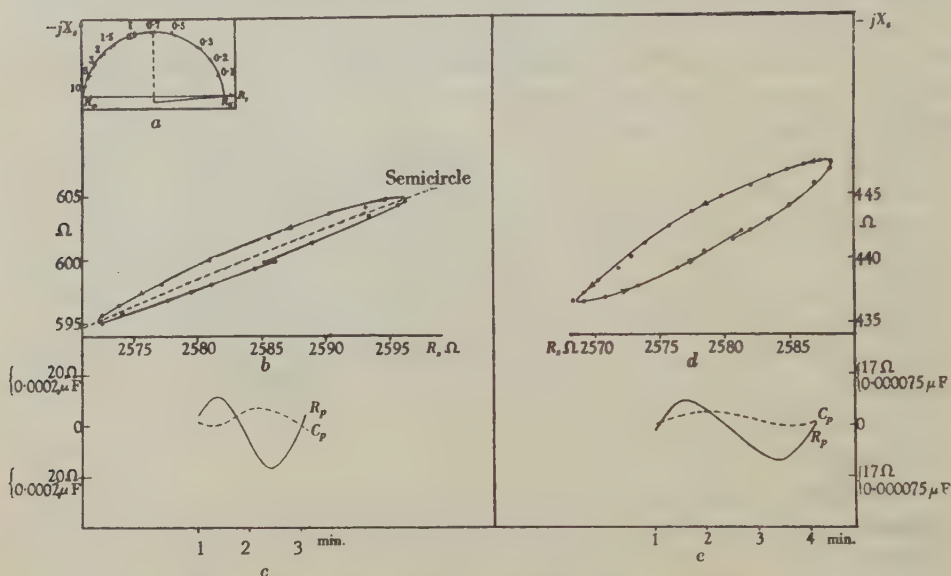
There is a possibility that the increase in conductivity of the distilled water round the egg at the beginning of the experiment is not entirely due to the outward diffusion of tap water from the perivitelline space, but to exosmosis from the egg proper. This possibility has not been investigated as it is not relevant to the experiments described in this paper.

The effect of chloretone. Phenyl urethane reversibly abolishes the impedance change (Rothschild, 1940). The experiments were carried out with relatively high concentrations of this substance ($6 \times 10^{-4} M$), and its inhibitory action can therefore be attributed to a general 'narcotic' effect. The reversibility of the inhibition suggests that at this concentration the narcotic inhibits some dehydrogenase system in the cell by non-specific adsorption. Experiments with $0.001 M$ -chloretone were carried out to see if the flavoprotein theory of inhibition (Quastel, 1943) had any relevance in the case of the metabolism associated with the rhythmical changes. The results were negative, although the cyanide experiments suggest that the cytochrome system is concerned in these oscillations.

Volume changes. Estimates of egg volumes were obtained by making planimeter measurements of egg areas on photographic enlargements. The measurements were accurate to about 2%. No rhythmical changes in area, nor therefore in volume, were observed.

Fine structure of the impedance cycle. The impedance changes can roughly be divided into two classes: first, a very regular type with a characteristic amplitude modulation every other cycle (Pl. 10a); and secondly, a much less regular type (Pl. 12a), in which the equivalent parallel resistance and capacitance do not go through balance at the same instant in time (i.e. they are out of phase), but in which again there is a typical impedance pattern. Previously (Rothschild, 1947a, b), the variation in impedance has only been resolved into its equivalent maximum and minimum resistive and reactive components. By use of the 'ellipse' technique the analysis can be extended to cover the whole cycle, and though it has not so far been possible to correlate the results of such analyses with the rhythmical egg movement, it seems worth while to record briefly the 'electrical' results of such experiments. Pl. 12a shows one of the second type of impedance variations, while from the ellipse traces of this impedance change, shown in Pl. 12b, it can be seen that the equivalent parallel resistance and capacitance are out of phase. Resistance balance occurs when the major ellipse axis is vertical, capacitance balance when the minor ellipse axis has its minimum width. If the resistance and capacitance changes are in phase, the ellipse degenerates into a line when the major ellipse axis is vertical; this is evidently not the case. The values of the equivalent parallel resistance and capacitance at any time in the cycle can be deduced from the ellipse traces, and

these can be converted into the equivalent series resistance and reactance form, from which the familiar circle diagram can be set up. Text-fig. 9*b* and *d* show the variation with time of the locus of the co-ordinate R_s, jX_s , at one frequency only, for regular and irregular types of impedance variation. These diagrams are, in fact, small parts of circle diagrams drawn on a very large scale ($c. \times 125$). Even in Text-fig. 9*b* there is a very slight suggestion of a resistance change in the system,



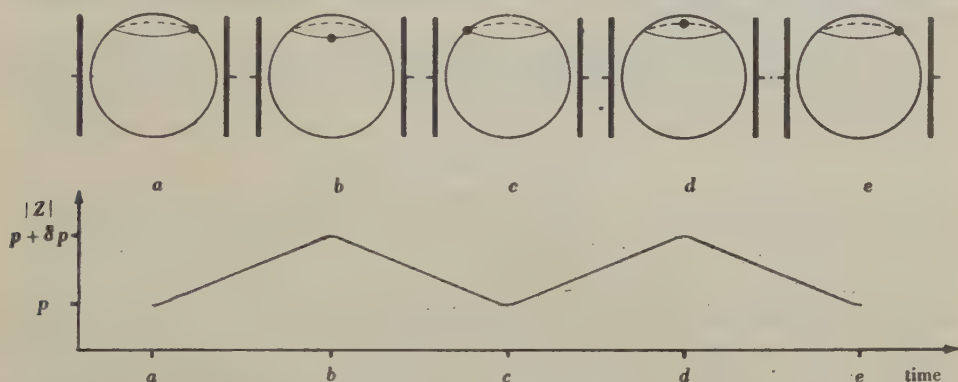
Text-fig. 9. *a*. Normal dynamic circle diagram for regular type of impedance change; R_0 , 3487 Ω . R_∞ , 2115 Ω . X_s at the characteristic frequency, 633 Ω . Frequencies in kilocycles. *b*. The same as *a*, but enlarged 125 times. The broken line is that part of the arc of the circle surrounded by a dotted line in *a*. In this time impedance locus at 1000 cycles, the points on the curve are 7.5 sec. apart. *c*. Variations in R_p and C_p (the resistance and capacitance equivalent to the egg in the conductivity cell) with time, from which *b* is obtained. Note that R_p and C_p reach their maxima and minima at the same time. The ordinates represent the tilt of the ellipse in the case of R_p and the width of the ellipse in the case of C_p . *d*. Time impedance locus for the irregular type of impedance change shown in Pl. 12. *e*. R_p and C_p variations from which *d* is obtained. Note different phase relationships.

which is not in phase with the main capacitance change, and is more marked in the irregular type (Text-fig. 9*d*). It is interesting that the main electrical difference between the two types lies in the degree to which the capacitance and resistance changes are out of phase, which can also be seen in the graphs showing the variation of the equivalent parallel resistance and capacitance with time, in Text-fig. 9.* Geometrically, the difference in the wave-form of the two types of impedance may be connected with the symmetry or asymmetry of the precession with respect to the two electrodes. This is discussed in more detail later.

* Alternative interpretations of the variations in the length of the 'loop' minor axis are possible. Analytically, the variation in loop area and tilt with the amplitude and phase relationships of R and C , when the latter are undergoing S.H.M., is somewhat complicated. As this is a purely electrical problem, it is not considered in this paper.

DISCUSSION

Correlation between egg movements and impedance changes. The egg can be considered as a sphere with uniform electrical properties, except for a cap, composed of the blastodisc and oil droplets, which has a different capacitance or dielectric constant from the rest of the egg. The vitelline membrane has a high resistance, which has not so far been measured, and a capacitance of the same order as that found in other biological material. The internal resistance of the egg is between 100 and 200 Ω cm. (Gray, 1932; Rothschild, 1946). When the egg is undergoing the rhythmical movements, the cap describes an approximately circular or elliptical path.* At any given moment the whole system has certain electrical properties, and is equivalent to some particular resistance and capacitance in parallel, at a particular



Text-fig. 10. Theoretical variations in impedance during precession of the egg. The egg drawings are purely diagrammatic. Ordinates, impedance (magnitude); abscissae, time.

frequency. Suppose that the electrodes are in the lateral position, and that the movement of the cap is exactly symmetrical with respect to the two electrodes; further, that the magnitude of the impedance of the system is p ohms at this moment, when the cap is in the position shown in Text-fig. 10*a*. When the cap has reached the position shown in Text-fig. 10*b*, the impedance of the system will be $p + \delta p$ ohms, where δp may be positive or negative. When, however, the cap reaches the position shown in Text-fig. 10*c*, the impedance of the system will be p ohms again, by virtue of the symmetry of the system egg + electrodes. For the same reason, the impedance will be $p + \delta p$ again when the cap is in the position shown in Text-fig. 10*d*, and of course will be p when the cap returns to its original position, having moved through one complete cycle. In these circumstances, as can be seen in Text-fig. 10, the result is that the impedance oscillation has double the frequency of the egg oscillation. If, however, the track of the cap is not exactly symmetrical with respect to both electrodes, the impedance records should reflect the frequency of the egg oscillations and this is what actually happens. The frequency of the amplitude

* Actually, the path is not circular or elliptical in the ordinary sense, because the movement is not in one plane. The movement does not take place on the surface of the sphere (the egg), but on the surface of an oblate spheroid, which is the surface enclosing the precessing sphere. What is seen through the microscope is the plane projection of the movement on this surface. A circular or bit will appear elliptical in these circumstances.

modulation, which is half the apparent frequency of the impedance cycle, is equal to the frequency of one complete egg oscillation.

When the electrodes are in the vertical plane, the cap is never symmetrically placed with respect to both electrodes. It alters its position relative to the electrode above the north pole of the egg, but its position relative to the south pole remains nearly constant. Consequently, the frequency doubling effect is very much reduced; and the impedance records correspond in frequency to that of the egg oscillation and are of smaller amplitude. As might be expected, there is a very slight effect due to the variation in the position of the cap relative to the south pole electrode. The possibility of the amplitude modulation (lateral electrodes) or the small 'harmonics' (vertical electrodes) being due to electrical changes in the vitelline membrane is discussed later.

The movement of the egg should not cause any change in the resistance of the system if Maxwell's equation for the resistance of a suspension of spheres is obeyed. This equation is

$$\frac{1 - r_1/r}{2 + r_1/r} = \rho \frac{1 - r_1/\bar{r}_2}{2 + r_1/\bar{r}_2},$$

where r_1 = resistivity of external medium, r = resistivity of whole system, $\bar{r}_2$ = resistivity of egg, ρ = volume concentration.

The position of the egg in the measuring apparatus does not come into this equation, and egg movements should not therefore cause any change in the resistance of the system unless the egg is heterogeneous with respect to its resistive properties. The existence of the polar cap of oil droplets suggests that if the egg (and therefore the cap) moves, the capacitance of the system will vary—and this is confirmed by the experiments.

Final proof that this interpretation is correct could be obtained by taking an egg which is not exhibiting any impedance cycle and moving it and the electrodes in such a way that the variation in orientation of the egg relative to the electrodes is similar to that which takes place when the electrodes are fixed in space and the egg precesses. The apparatus shown in Text-fig. 3 was designed for this purpose, but experiments with it, though tending to confirm this interpretation, were not entirely successful. Superficially it may seem that this method of confirming the electrical effects of the egg precession is rather roundabout, and that the same result could be achieved by keeping the electrodes fixed and artificially making the egg precess. This is impossible because of the property the egg has of always orienting itself so that the blastodisc and the oil droplets are at the north pole. For example, if an egg in its chorion is turned upside down so that the blastodisc is at the south pole, rotation occurs at once and the blastodisc comes back to the north pole. This phenomenon, which is common to many fish eggs, has caused certain authors to liken fish eggs to 'Stehaufmännchen' (Kuhl, 1939).

In a previous paper (Rothschild, 1946), it was shown experimentally that Maxwell's equation does not hold exactly for a system of this type, and it can also be shown on theoretical grounds that this is so. Correspondingly, it is found that very small changes in resistance do occur when an egg is moved about in a conductivity cell.

Possibly the small changes in resistance that were described in the 'Results' section under 'Fine structure of the impedance cycle' (in the case of the regular type of impedance change) may in part be due to such deviations from Maxwell's equation.

The question arises as to whether the recorded impedance changes are entirely due to the 'electro-geometrical' changes discussed above, or to electrical changes in the vitelline membrane itself. The capacitance change is almost certainly in the first category. It is possible that the small resistance changes in Text-fig. 9 might be due to resistance changes in the vitelline membrane, but there is negligible evidence in support of this possibility. If the movements of the egg are due to some contractile property of the vitelline membrane, and if the contraction of the vitelline membrane is associated with changes in its resistance, the concomitant impedance changes might be expected to occur at the *beginning* of each impedance cycle, or at any rate not all the way through the egg cycle. This is exactly what happens, if we care to interpret the impedance changes shown in Pl. 10 in the appropriate way. Superimposed on the impedance changes due to the dielectric heterogeneity of the egg surface, there is, according to this view, some other change which increases the amplitude of the impedance variation; the effect has a significantly smaller duration than the whole cycle. I do not think that this complicated interpretation is justified at present. The simplest hypothesis is that the impedance changes are caused by the dielectric heterogeneity and movement of the egg, and that the amplitude modulations are due to the precession not being symmetrical with respect to both electrodes; but it is difficult to establish this with complete certainty.

Causes of the precession. In other fish eggs, similar movements have been correlated with waves of contraction in the vitelline membrane, passing over the whole egg (Ransom, 1867; Yamamoto, 1933). No such contraction waves have as yet been observed in unfertilized trout eggs. The movements must presumably be due to periodic changes in the centre of gravity of the egg, of a peculiarly regular nature.

Metabolism

Investigations into the metabolism of the egg during the rhythmical changes are made somewhat difficult by the impermeability of the vitelline membrane and even perhaps by the large amount of yolk within the vitelline membrane, in which diffusion may be slow. The fact that cyanide reversibly reduces the frequency and amplitude of the changes, and in sufficiently high concentrations inhibits it altogether, indicates that the cytochrome system is involved; but we cannot conclude, as has been done for analogous reasons in the case of ciliary movement (Gray, 1928), that because cyanide exerts its effect slowly, the cytochrome system is involved in a resynthesis stage in the metabolic cycle and not in the breakdown of the energy-producing compound. The same difficulties would apply to the interpretation of experiments carried out in anaerobic conditions, because of the possibility of oxygen reserves in the yolk. The experiments with phlorizin and NaF, which can be interpreted as indicating that the changes are not associated with any glycolytic metabolism, are in the same category.

There are two ways in which the egg movements seem to differ from ciliary movement. First, although a decrease in the pH of the external medium has a marked and immediate effect on the oscillations, recovery sets in later. The influence of pH changes has not been examined in detail, and such experiments would be of some interest. The experiments carried out at low pH's were a by-product of those with sodium azide. Secondly, the presence or absence of Ca^{++} in the external medium is without effect. This might suggest that calcium does not play a part in the reaction responsible for the egg movements; but this seems unlikely as calcium lack (i.e. distilled water) has a marked effect on the eggs, making them much more sensitive to mechanical shock than when in the normal environment. Equally, $\text{M}/25\text{-CaCl}_2$ in tap water has no inhibitory effect, which disposes of the possibility that the changes are comparable to those that occur in nerves when the Ca^{++} concentration of the medium is reduced.

The origin and significance of these egg movements may be elucidated by studying the effects of other chemicals on them. The influence of veratrine, narcotics at different concentrations, CO in the dark and light, Mg^{++} , K^+ , and H^+ would be of interest.

Temperature

These experiments were carried out to obtain data for comparison with other rhythmical biological phenomena, rather than to try to interpret the mechanism of the changes by attaching some significance to the absolute value of the Q_{10} or temperature characteristic. There is a marked similarity between the temperature characteristic of the egg oscillation and that of ciliary movement. This is evident if we compare the results described in this paper with those obtained and described by Gray (1928). Crozier's description of Gray's data on the temperature characteristics of ciliary movement and their O_2 consumption gives a somewhat different picture, however, as he states that there is a significant 'break' at 15° , above which $\mu = 11,800$ and below which $\mu = 16,700$ (Crozier, 1924a). From this it might be concluded that the mechanism responsible for the variation in the frequency of egg oscillations with temperature is different from that responsible for the variations in ciliary movement with temperature, because no break occurs in the former at 15° . Such a conclusion is hardly justifiable when it is remembered that Gray found no break at 15° and drew a straight line, with a correspondingly smaller slope, through the points obtained at different temperatures.

A convenient table for comparison of the μ values for different biological processes is to be found in the Appendix, 'Index to temperature characteristics', in Barnes's *Textbook of General Physiology* (1937). In this table protoplasmic streaming (cyclosis) is said to have a temperature characteristic of '4700-10,300', amoeboid movement 10,800, and the pulsation of smooth muscle in rabbit intestine 8000 and 16,150. The pulsation of contractile vacuoles can be shown to have a mean value of 16,400. Trout-egg development is said to have μ equal to 24,500, while the first cleavage of the frog's egg has μ equal to 10,800 or 21,900. From these data one might conclude that it is unwise to dogmatize about the nature of some biological process solely from a consideration of the variation

in velocity of the process with temperature. Yet Yamamoto (1931, p. 161), who investigated what are undoubtedly similar movements in the fertilized egg of the Japanese medaka *Oryzias latipes*, and found temperature characteristics of 10,000–11,000, 21,000–22,000 and 27,000–29,000, between 7 and 39° C., concludes that the movements ‘unquestionably differ from those vital activities known to be associated with oxidative processes...’, and that the temperature characteristics ‘agree well with those heretofore obtained for the rate of development or growth phenomena’; while, as mentioned in the Introduction, Needham implies that these changes are in some way a result of fertilization and that their function is to stir up the yolk for the benefit of the embryo. These views are not easy to reconcile with the fact that the oscillations occur in unfertilized eggs, unless we postulate that *all* unfertilized fish eggs sustain a parthenogenetic stimulus when placed in fresh water. I have tentatively suggested that this may be the case (Rothschild, 1935; Hubbard & Rothschild, 1939), and Trifonowa’s experiments (1934) show that the eggs of fresh-water fish are somewhat liable to be parthenogenetically activated—though abortively—when placed in fresh water. There is some evidence, which I hope to publish elsewhere, that this is so in the case of frogs’ eggs; but the data on temperature characteristic recorded in this paper neither support nor controvert this possibility in the case of the eggs of trout.

There is a further point about these egg movements which should be mentioned. It has been tacitly assumed that the *whole* egg precesses, or that after being turned upside down the *whole* egg rotates until the blastodisc is at the north pole. The same applies to the frog’s egg, which rotates within the perivitelline space so that the white pole is at the bottom of the egg. If the egg as a whole did not rotate or precess, but only the vitelline membrane moved, the optical results would be the same, and it is not easy to distinguish between these alternatives. Ancel & Vintemberger (1933) have suggested that something of this sort happens in the frog’s egg.

SUMMARY

1. Accelerated cine-photographs show that the unfertilized trout egg exhibits rhythmical movements within its chorion after immersion in tap water. The movement can be described as a periodic axial perturbation or ‘precession’, similar to that which takes place in a slowing-down top. The egg does not, however, spin at the same time. The frequency of the motion is about 3·4 min. at 16·8° C.

2. There is a strict correlation between the frequency of the egg precession and the frequency of the impedance changes that are observed when the egg is placed in a conductivity cell in an alternating current bridge. The impedance changes exhibit a characteristic ‘impedance pattern’, the frequency of which is identical with that of the precession.

3. The oscillations are neither initiated nor affected by the measuring current.

4. The question as to whether the electrical changes are entirely caused by the dielectric heterogeneity and precession of the egg, or whether they are partly due

to changes in the vitelline membrane, associated with the egg movements, is discussed. The former is the simpler hypothesis, and there is some evidence in support of this interpretation from impedance measurements made with the electrodes in different planes relative to the egg. It was difficult to reach a firm decision on this point because the electrical method of recording the changes is more sensitive and accurate than existing optical methods.

5. Examination of the fine structure of the impedance cycle, by means of the ellipse technique and enlarged time-impedance loci at particular frequencies, shows that there is a very small resistance change associated with the impedance oscillations, which is not in phase with the capacitance change. The possibility of these resistance changes being due to variations in the position of the egg relative to the measuring electrodes, combined with small deviations from Maxwell's formula, or to resistive heterogeneity in the vitelline membrane, or to changes in the resistance of the vitelline membrane, is discussed.

6. The variation of frequency with temperature has been investigated. In one typical run, the temperature characteristic was 15,200 which corresponds to a Q_{10} of 2.47. The average value of the temperature characteristic for the whole season was 16,200. There are no obvious 'breaks' in the curve relating log velocity to the reciprocal of the absolute temperature; the curve obeys Arrhenius's equation except at temperatures above 18° C., when irreversible changes tend to set in. There is no very convincing reason for associating this temperature characteristic with developmental processes, particularly as measurements are made on unfertilized eggs. On the other hand, the possibility of *all* unfertilized eggs undergoing abortive parthenogenetic activation on immersion in tap or stream water is discussed.

7. Sodium cyanide (M/300, M/500, M/100 in tap water, pH adjusted) reversibly reduces the frequency and amplitude of the impedance changes, which suggests that cytochrome is concerned in the metabolic system responsible for the egg movements. The frequency is more sensitive to cyanide than the amplitude. There are indications that this compound has some effect on the electrical properties of the vitelline membrane, but this has not been systematically examined. Sodium azide, which has no effect at pH 7.4, cannot be used at pH 6.5 because this concentration of H^+ in the external medium has a marked, though not permanent, effect on the egg movements and impedance changes.

8. Neither phlorizin (M/100, M/200, M/500 in tap water, pH not adjusted), nor sodium fluoride (M/25, pH not adjusted) have any effect on the egg movements or the impedance cycle. This suggests that the energy for these changes is not glycolytic in origin; but there is a possibility that the molecules of these inhibitors do not penetrate the vitelline membrane.

9. The action of the inhibitors does not as yet permit any comparison to be made with ciliary or amoeboid movement, or with muscular contraction, as the experiments do not enable a distinction to be made between the utilization of oxygen in the breakdown phase or the resynthesis phase of the metabolic cycle.

10. The following substances have no effect on egg movements or impedance changes:

(a) Laboratory-distilled water. There is a possibility that distilled water affects the properties of the vitelline membrane, in the sense that some exosmosis of electrolytes occurs when the egg is in the medium. This has not been systematically examined as it has no direct bearing on the egg oscillations.

(b) $M/25\text{-CaCl}_2$ in tap water.

(c) 0.001M -chloretone in tap water.

11. There is no appreciable periodic change in egg volume.

REFERENCES

- ANCEL, P. & VINTEMBERGER, P. (1933). *C.R. Soc. Biol., Paris*, **114**, 1035.
 ARVANITAKI, A. (1939). *Arch. int. Physiol.* **49**, 209.
 BARNES, T. C. (1937). *Textbook of General Physiology*. Philadelphia.
 COLE, K. S. (1928). *J. Gen. Physiol.* **12**, 37.
 COLE, K. S. (1935). *J. Gen. Physiol.* **18**, 877.
 COLE, K. S. (1938). *Nature, Lond.*, **141**, 79.
 COLE, K. S. & CURTIS, H. J. (1938). *J. Gen. Physiol.* **22**, 37.
 COLE, K. S. & CURTIS, H. J. (1939). *J. Gen. Physiol.* **22**, 649.
 COLE, K. S. & MARMONT, C. (1942). *Fed. Proc.* **1**, 15.
 CROZIER, W. J. (1924*a*). *J. Gen. Physiol.* **7**, 123.
 CROZIER, W. J. (1924*b*). *J. Gen. Physiol.* **7**, 189.
 FRICKE, H. & CURTIS, H. J. (1934). *J. Gen. Physiol.* **18**, 821.
 GRAY, J. (1928). *Ciliary Movement*. Cambridge.
 GRAY, J. (1932). *J. Exp. Biol.* **9**, 277.
 HODGKIN, A. L. & HUXLEY, A. F. (1946). *Nature, Lond.*, **158**, 376.
 HUBBARD M. J. & ROTHSCHILD, LORD (1939). *Proc. Roy. Soc. B*, **127**, 510.
 KEILIN, D. (1936). *Proc. Roy. Soc. B*, **121**, 165.
 KUHL, W. (1939). *Z. wiss. Zool.* **152**, 305.
 NEEDHAM, J. (1942). *Biochemistry and Morphogenesis*. Cambridge.
 QUASTEL, J. H. (1943). *Trans. Faraday Soc.* **39**, 1.
 RANSOM, W. H. (1867). *Philos. Trans.* **157**, 431.
 ROTHSCHILD, N. M. V. (1935). Dissertation, Trinity College, Cambridge.
 ROTHSCHILD, LORD (1940). *Nature, Lond.*, **145**, 744.
 ROTHSCHILD, LORD (1946). *J. Exp. Biol.* **23**, 77.
 ROTHSCHILD, LORD (1947*a*). *Nature, Lond.*, **159**, 134.
 ROTHSCHILD, LORD (1947*b*). *J. Exp. Biol.* **23**, 267.
 RUSCONI, M. (1840). *Arch. Anat. Physiol.* Heft 11, 185.
 TRIFONOWA, A. (1934). *Acta zool., Stockh.*, **15**, 183.
 WINTREBERT, P., PAINLEVÉ, J. & YUNG KO-CHING (1929). *C.R. Acad. Sci., Paris*, **189**, 208.
 YAMAMOTO, T. (1931). *J. Fac. Sci. Imp. Univ. Tokyo*, Sec. IV, Zool. **2** (part 3), 153.
 YAMAMOTO, T. (1933). *J. Fac. Sci. Imp. Univ. Tokyo*, Sec. IV, Zool. **3** (part 2), 111.
 YAMAMOTO, T. (1934). *J. Fac. Sci. Imp. Univ. Tokyo*, Sec. IV, Zool. **3**, 275.
 YAMAMOTO, T. (1940). *Annot. zool. jap.* **19**, 69.

EXPLANATION OF PLATES

PLATE 9

Photographs, at intervals of 49 sec., of one complete egg cycle. Note that in ii and v, though the egg appears to be in the same position relative to the chorion, the oil globules under the proximal egg surface are in focus in ii, but out of focus in v. This means that if the camera had been in the x - z , instead of the y - z plane, the egg would not have been in the same position relative to the chorion in ii and v.

PLATE 10

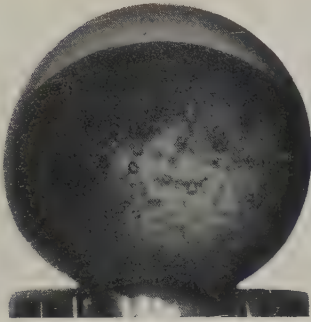
a. Normal impedance change. $T^{\circ}\text{C. } 16.8$. Time-marker, minutes. The dotted line shows one 'complete' impedance change. *b.* Measurements of impedance in different planes relative to the egg. $T^{\circ}\text{C. } 16.8$. Time-marker, minutes. i, electrodes in normal position; ii, electrodes in vertical position. *c.* Effect of current interruptions. $T^{\circ}\text{C. } 16.8$. No interruption, time-marker, minutes; interruption lasting 5 sec.; interruption lasting 10 sec. *d.* Effect of pH. i, in tap water, pH 7.3; ii, after 6 min. in tap water, pH 6.3. $T^{\circ}\text{C. } 16.8$. Time-marker, minutes.

PLATE 11

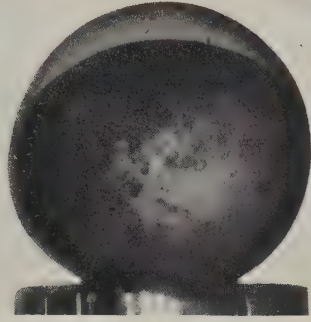
Effect of sodium cyanide (M/1000 in tap water). $T^{\circ}\text{C. } 16.8$. Time-marker, minutes. i, before treatment; ii-v, 28, 78, 170 and 225 min. after immersion; vi, vii, recovery in tap water, after 3 and 15 hr.

PLATE 12

a. Irregular type of impedance change: time-marker, minutes. $T^{\circ}\text{C. } 16.8$. *b.* Ellipse traces for *a* showing R_p (tilt) and C_p (width) out of phase.



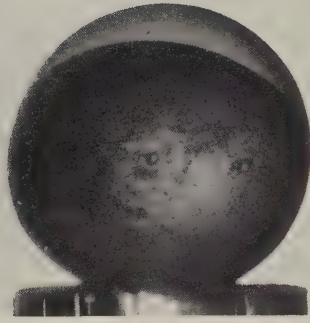
i



ii



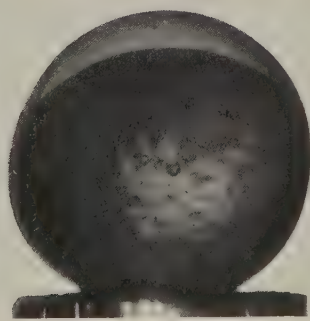
iii



iv

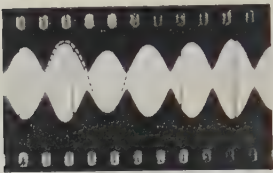


v

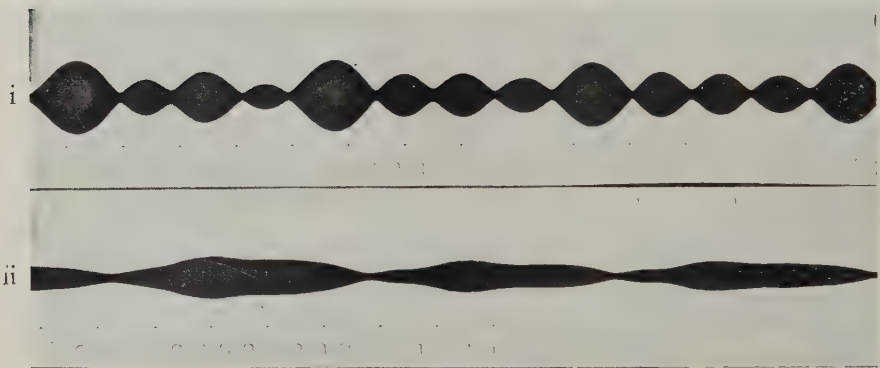


vi

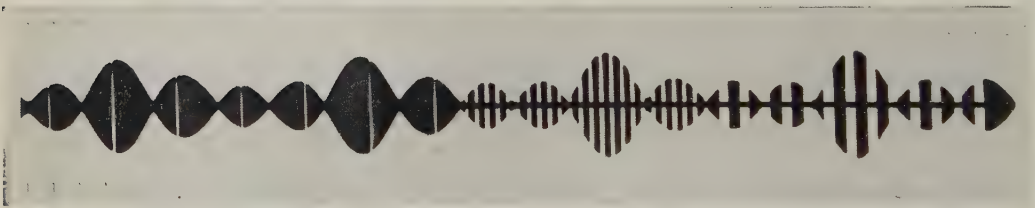
ROTHSCHILD—RHYTHMICAL IMPEDANCE CHANGES IN THE
EGG OF THE TROUT



a



b

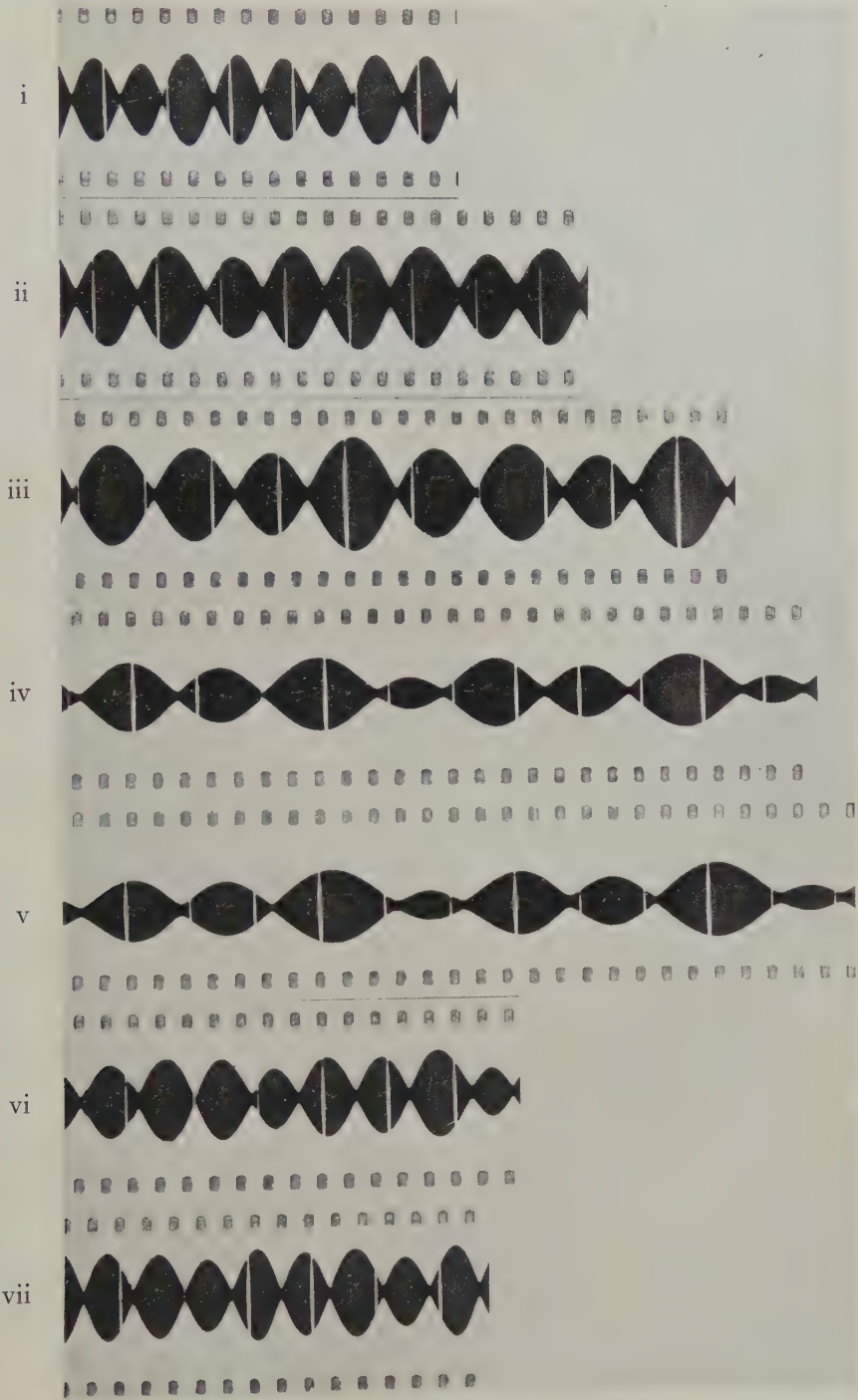


c

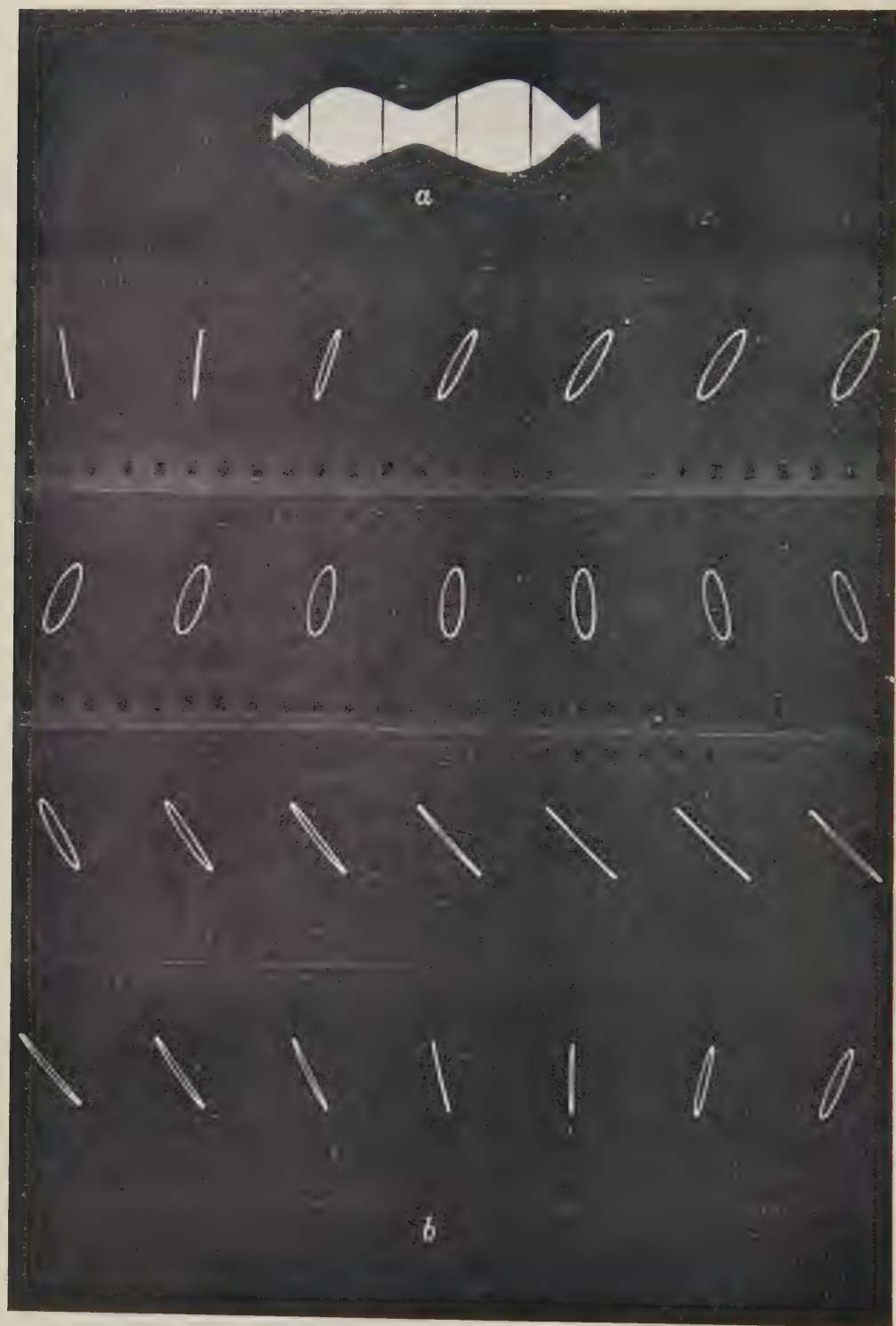


d

ROTHSCHILD—RHYTHMICAL IMPEDANCE CHANGES IN THE EGG OF THE TROUT



ROTHSCHILD—RHYTHMICAL IMPEDANCE CHANGES IN THE
EGG OF THE TROUT



ROTHSCHILD—RHYTHMICAL IMPEDANCE CHANGES IN THE
EGG OF THE TROUT

INDEX OF SUBJECTS

A

- Aedes aegypti* (L.):
Observations on the effect of microclimate
on biting by (LUMSDEN) 361

Agriotes:
See Wireworm

Aphelocheirus:
See Plastron respiration

B

- Bedbug:
Preliminary studies on the nutritional re-
quirements of the (*Cimex lectularius* L.)
and the tick *Ornithodoros moubata* Murray
(MEILLON and GOLBERG) 41

C

- Calandra granaria* Linn.:
The influence of temperature and humidity
on instar length in (EASTHAM and SEGROVE)
79

Cellulose:
The digestion of, by sheep. The extent of
cellulose digestion at successive levels of the
alimentary tract (GRAY) 15

Central nervous system:
Investigations on the regional determi-
nation of the (NIEUWKOOP) 145

Chemoreceptors:
of the wireworm (*Agriotes* spp.) and the
relation of activity to chemical constitution
(CROMBIE and DARRAH) 95

Chironomus:
On the function of haemoglobin in, after
oxygen lack (WALSHE) 329

Cimex lectularius L.:
See Bedbug

Crustacea:
Local factors and regeneration in (NEEDHAM)
220

D

Diphyllobothriidae:
Aseptic cultivation of larval, *in vitro*
(Studies on tapeworm physiology, III)
(SMYTH) 374

E

Electro-magnetic mixer:
for manometric experiments (LASER and
ROTHSCHILD) 387

F

Fatty acids:
The absorption of volatile, from the rumen
(GRAY) 1
— The analysis of mixtures of (GRAY) 11

G

Glycogen:
Distribution of, in the developing salmon
(*Salmo salar* L.) (DANIEL) 123
Greenfinch:
Experimental modification of the sexual
cycle of the (DAMSTÉ) 20

H

Haemoglobin:
On the function of, in *Chironomus* after
oxygen lack (WALSHE) 329
— The function of, in *Tanytarsus* (Chirono-
midae) (WALSHE) 343

M

Mallard:
Seasonal cyclical changes in the thymus of
the (HÖHN) 184

Microclimate:
Observations on the effect of, on biting
by *Aedes aegypti* (L.) (Dipt., Culicid.)
(LUMSDEN) 361

Micro-respirometer, metal:
of the Barcroft type suitable for small insects
and other animals (CRISP and THORPE) 304

Mosquitoes:
Visual responses of, artificially rendered
flightless (RAO) 64

O

Ocellus, insect:
The function of the (PARRY) 211

Ornithodoros moubata Murray:
See Bedbug

Oyster muscle:
The succinoxidase system in (HUMPHREY)
352

P

Plastron respiration:
Studies on:
— I. The biology of *Aphelocheirus* [Hemiptera,
Aphelocheiridae (Naucoridae)] and the
mechanism of plastron retention (THORPE
and CRISP) 227
— II. The respiratory efficiency of the plastron
in *Aphelocheirus* (THORPE and CRISP) 270
— III. The orientation responses of *Aphelo-*
cheirus [Hemiptera, *Aphelocheiridae* (Nau-
coridae)] in relation to plastron respiration;
together with an account of specialized
pressure receptors in aquatic insects
(THORPE and CRISP) 310

Pressure receptors in aquatic insects:
See Plastron respiration

Pygosteus pungitius L.:
The reactions of, to toxic solutions (JONES)
110

R

Rabbits:

Studies on sterility and prenatal mortality in wild. Part III. The loss of ova before implantation (BRAMBELL and MILLS) 192

Regeneration in Crustacea:

Local factors and (NEEDHAM) 220

Rhythmical impedance:

changes in the egg of the trout. III (ROTHS-CHILD) 390

S

Salmon (*Salmo salar* L.):

Distribution of glycogen in the developing (DANIEL) 123

Sexual cycle of the greenfinch:

Experimental modification of the (DAMSTÉ) 20

Sterility and prenatal mortality:

Studies on, in wild rabbits. III. The loss of ova before implantation (BRAMBELL and MILLS) 192

Succinoxidase system:

in oyster muscle (HUMPHREY) 352

T

Tanytarsus (Chironomidae):

The function of haemoglobin in (WALSHE) 343

Tapeworm physiology:

Studies on. III. Aseptic cultivation of larval diphylobothriidae *in vitro* (SMYTH) 374

Temperature and humidity:

The influence of, on instar length in *Calandra granaria* Linn. 79

Tick:

See Bedbug

Toxic solutions:

The reactions of *Pygosteus pungitius* L. to (JONES) 110

Trout egg:

Rhythmical impedance in the, III (ROTHS-CHILD) 390

W

Wireworm:

The chemoreceptors of the (*Agriotes* spp.), and the relation of activity to chemical constitution (CROMBIE and DARRAH) 95

INDEX OF AUTHORS

B

- Brambell, F. W. Rogers and Mills, Ivor H. Studies on sterility and prenatal mortality in wild rabbits. Part III. The loss of ova before implantation 192

C

- Crisp, D. J. and Thorpe, W. H. A metal micro-respirometer of the Barcroft type suitable for small insects and other animals 304
— See also THORPE and CRISP
Crombie, A. C. and Darrah, J. H. The chemoreceptors of the wireworm (*Agriotes* spp.) and the relation of activity to chemical constitution 95

D

- Damsté, P. H. Experimental modification of the sexual cycle of the greenfinch 20
Daniel, R. J. Distribution of glycogen in the developing salmon (*Salmo salar* L.) 123
Darrah, J. H. See CROMBIE and DARRAH

E

- Eastham, L. E. S. and Segrove, F. The influence of temperature and humidity on instar length in *Calandra granaria* Linn. 79

G

- Golberg, Leon. See MEILLON and GOLBERG
Gray, F. V. The absorption of volatile fatty acids from the rumen 1
— The analysis of mixtures of fatty acids 11
— The digestion of cellulose by sheep. The extent of cellulose digestion at successive levels of the alimentary tract 15
Gray, J. and Lissman, H. W. The effect of labyrinthectomy on the co-ordination of limb movements in the toad 36

H

- Höhn, E. O. Seasonal cyclical changes in the thymus of the mallard 184
Humphrey, G. F. The succinoxidase system in oyster muscle 352

J

- Jones, J. R. Erichsen. The reactions of *Pygosteus pungitius* L. to toxic solutions 110

L

- Laser, H. and Rothschild, Lord. An electromagnetic mixer for manometric experiments 387
Lissman, H. W. See GRAY and LISSMAN
Lumsden, W. H. R. Observations on the effect of microclimate on biting by *Aedes aegypti* (L.) (Dipt., Culicid.) 361

M

- Meillon, Botha de and Golberg, Leon. Preliminary studies on the nutritional requirements of the bedbug (*Cimex lectularius* L.) and the tick *Ornithodoros moubata* Murray 41

Mills, Ivor H. See BRAMBELL and MILLS

N

- Needham, A. E. Local factors and regeneration in Crustacea 220
Nieuwkoop, P. D. Investigations on the regional determination of the central nervous system 145

P

- Parry, D. A. The function of the insect ocellus 211

R

- Rao, T. Ramachandra. Visual responses of mosquitoes artificially rendered flightless 64
Rothschild, Lord. Rhythmical impedance changes in the egg of the trout. III 390
— See also LASER and and ROTHSCHILD

S

- Segrove, F. See EASTHAM and SEGROVE
Smyth, J. D. Studies on tapeworm physiology. III. Aseptic cultivation of larval diphylobothriidae *in vitro* 374

T

- Thorpe, W. H. and Crisp, D. J. Studies on plastron respiration:
— I. The biology of *Aphelocheirus* [Hemiptera, Aphelocheiridae (Naucoridae)] and the mechanism of plastron retention 227
— II. The respiratory efficiency of the plastron in *Aphelocheirus* 270
— III. The orientation responses of *Aphelocheirus* [Hemiptera, Aphelocheiridae (Naucoridae)] in relation to plastron respiration; together with an account of specialized pressure receptors in aquatic insects 310

W

- Walshe, Barbara M. On the function of haemoglobin in *Chironomus* after oxygen lack 329
— The function of haemoglobin in *Tanytarsus* (Chironomidae) 343

